MicroRNAs and Vascular Remodeling in Pulmonary Hypertension

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Matthias Brock

aus

Deutschland

Promotionskomitee:

Prof. Dr. Max Gassmann (Vorsitz)

Prof. Dr. Steffen Gay (Leitung der Dissertation)

Prof. Dr. François Verrey

Zürich, 2012

Summary (English)

Pulmonary arterial hypertension (PAH) is a devastating condition defined by the sustained elevation of pulmonary vascular resistance that rapidly leads to right heart failure and death when left untreated. The pathogenesis of PAH is characterized by vascular remodeling and, among others, the dysregulated expression of the bone morphogenetic protein receptor type II (BMPR2). Mutations in the gene encoding BMPR2 were identified in more than 70% of hereditary PAH cases and, moreover, reduced expression levels of BMPR2 were also found in patients with non-genetic PAH and in other forms of pulmonary hypertension (PH) as well as in animal models mimicking the disease. The different WHO classes of PH are provided as Table 1 within the introduction. It is largely accepted that the loss of BMPR2 represents a key event in the pathogenesis of PAH, and, moreover, might constitute a final common pathway in other forms of PH by enhancing proliferation and apoptosis resistance of cells surrounding the small pulmonary arteries eventually resulting in vascular remodeling. So far, the mechanisms leading to reduced expression of BMPR2 remained unclear.

The focus of this PhD thesis was set on the identification of mechanisms that control the expression of BMPR2 in PAH. Since microRNAs (miRNAs) have emerged as novel repressors of gene expression, it was hypothesized that miRNAs might play a role in the modulation of BMPR2. Using a database for computational prediction of miRNA-target interactions several miRNAs encoded by the microRNA cluster 17/92 (miR-17/92) were retrieved as potential regulators of BMPR2. The first part of my thesis provides evidence that the expression of BMPR2 is directly regulated by two miRNAs (miR-17 and miR-20a) derived from miR-17/92. Moreover, stimulation experiments in endothelial cells revealed increased expression of miR-17/92 by interleukin (IL-) 6, a cytokine involved in the pathogenesis of PAH. The context of IL-6, miR-17/92 and BMPR2 was further demonstrated by showing that persistent activation of the IL-6 pathway led to repressed protein expression of BMPR2.

Consistent with the hypothesis of a miR-17/92-mediated reduction of BMPR2, a recent study showed increased expression of miR-17 and miR-20a in the lungs of PH animals. Therefore, to address the pathophysiological relevance of miR-20a *in vivo*, we investigated whether specific inhibition of miR-20a by small antisense RNA oligonucleotides (antagomiRs) could restore functional levels of BMPR2 and, in turn, might prevent pulmonary arterial vascular remodeling. Presented in the second part of this thesis, we found that antagonizing miR-20a in

the hypoxia-induced mouse model for pulmonary hypertension upregulated the expression levels of BMPR2 and, moreover, significantly reduced wall thickness and luminal occlusion of small pulmonary arteries. Consistently, the proliferation of human pulmonary arterial smooth muscle cells *in vitro* was found to be reduced upon transfection with antagomiR-20a. These data emphasize that treatment with antagomiR-20a restores the expression of BMPR2 in pulmonary arteries and prevents the development of vascular remodeling. Therefore, the application of antagomiR-20a could be a promising approach for a causative therapy of PH.

Apart from BMP signaling, it was shown that the IL-6 signaling cascade significantly contributes to the maintenance of the vasculature. Along this line, lung-specific IL-6-over expressing transgenic mice spontaneously developed PH accompanied by vasculopathic changes. Moreover, the serum concentration of IL-6 was also found to be increased in patients with PAH. Regarding the importance of IL-6 and miR-17/92 for PH, we further investigated the interplay of miR-17/92 and IL-6 signaling and its impact on an inflammatory model, the acute-phase response. In the last part of this thesis, we provide evidence that IL-6 upregulated the expression of miR-17/92 in different cell types and that one miRNA derived from miR-17/92, miR-18a, was a potent activator of IL-6 signaling by targeting an endogenous repressor of this pathway. These data reveal for the first time a miRNA mediated positive feedback loop of IL-6 signal transduction and thus contribute to the understanding of the complex regulatory mechanisms within the signal transduction of IL-6.

Zusammenfassung (Deutsch)

Die pulmonale Hypertonie (PH) ist der Überbegriff für verschiedene Krankheitsbilder, welche in einer signifikanten Erhöhung des Blutdrucks im Lungenkreislauf resultieren und auf Grund des vermehrten rechtsventrikulären Afterloads letztendlich zum Rechtsherzversagen führt. Die chronisch Drucksteigerung in der Lungenblutbahn wird im Wesentlichen durch drei pathogenetische Ereignisse verursacht: eine Mikrothrombosierung der Gefässbahn, eine übermässige Vasokonstriktion, sowie durch einen fibrotischen Gefässumbau („Remodeling“). Bei letzterem scheint das Gen BMPR2 (bone morphogenetic protein – Rezeptor Typ II) eine entscheidende Rolle zu spielen. In ca. 70% aller Patienten mit einer familären Form der pulmonal-arteriellen Hypertonie (PAH) ist das BMPR2-Gen durch DNA-Mutationen inaktiviert. Interessanterweise wurde aber auch in nicht-vererbaren Formen der PAH sowie in anderen Formen der PH eine verminderte Expression von BMPR2 beschrieben. Auch wenn die Rolle von BMPR2 nicht definitiv geklärt ist, so könnte die reduzierte Expression von BMPR2 den Gefässumbau im Wesentlichen mitverursachen, da durch den Verlust von BMPR2 sowohl die Proliferation als auch die Apoptose-Resistenz von Endothelzellen und glatten Gefässmuskelzellen erhöht wird. Die molekularbiologischen Mechanismen, die zu einer verringerten Oberflächenexpression von BMPR2 in den nicht vererbaren Formen der PH führen, sind jedoch bisher unbekannt.

Der Schwerpunkt meiner Doktorabeit war die Erforschung der Regulation der BMPR2-Expression im Kontext der PH. Auf Grund von Studien, die eine Inhibierung des Translations-Prozesses von BMPR2 während der Entwicklung von PH implizierten, untersuchte ich die post-transkriptionale Regulation von BMPR2 durch so genannte microRNAs (miRNAs). miRNAs sind kleine, nicht proteinkodierende RNA Moleküle, denen ein grosses Potential im Feld der post-transkriptionalen Genregulation zugesprochen wird. Mit Hilfe von Computerprogrammen, die Sequenzähnlichkeiten zwischen miRNAs und potentiell miRNA-regulierten Genen analysieren, konnte ich den miRNA cluster miR-17/92 als möglichen Regulator von BMPR2 identifizieren. Weiterführende Experimente bestätigten, dass zwei miRNAs (miR-17 und miR-20a) aus dem miR-17/92 cluster direkt und spezifisch die Expression von BMPR2 kontrollieren. Desweiteren konnte gezeigt werden, dass nach Stimulation von pulmonalen Endothelzellen mit Interleukin (IL)-6, dessen Serumspiegel bei PAH-Patienten erhöht ist, die Expression von miR-17/92 erhöht war und dass eine

Überaktivierung der IL-6 Signalkaskade zu einer Verminderung der BMPR2-Expression führte.

Um die physiologische Bedeutung von miR-20a für den Krankheitsverlauf der PH zu untersuchen, verwendete ich das Mausmodell der Hypoxie-induzierten PH, in dem die Expression vom miR-20a gezielt durch die Applikation von komplementären, anti-sense-orientierten RNA Molekülen („AntagomiRs“) ausgeschaltet wurde. In dieser Studie konnte gezeigt werden, dass die Exposition der Tiere gegenüber chronisch-hypoxischen Konditionen zu einer Verringerung der BMPR2-Expression in der Lunge führte. Die Antagonisierung von miR-20a konnte dabei die BMPR2-Expression auf das Niveau der normoxischen Kontrollgruppe normalisieren. Desweiteren zeigten physiologische Untersuchungen, dass sowohl die für PH charkteristische rechtsventrikuläre Hypertrophie als auch der Gefässumbau von pulmonalen Arteriolen durch Inhibierung von miR-20a signifikant reduziert wurde. Diese Daten untermauern die Hypothese, dass miR-20a ein wichtiger Modulator für die Expression von BMPR2 und somit ein entscheidender Faktor im Prozess des Gefässumbaus ist. Daher könnten Medikamente, die auf eine Inhibierung von miR-20a abzielen, einen neuen vielversprechenden Ansatz zur Behandlung von PH darstellen.

Im letzten Teil meiner Doktorarbeit untersuchte ich das Wechselspiel von IL-6 und dem miR-17/92 cluster. Am Modell der hepatischen Akut-Phase-Reaktion, die eine sehr gut beschriebene Form der IL-6 Signalkaskade repräsentiert, konnte gezeigt werden, dass miR-17/92 Teil eines positiven Feedback-Loops in der Regulation des IL-6-Signalweges ist. Insbesondere konnte ich dabei zeigen, dass miR-18a (ein Mitglied des miR-17/92 clusters) die Expression von PIAS3, einen Repressor des inflammatorischen IL-6-Signalweges, verminderte und somit die hepatische Akut-Phase-Reaktion verstärkte. Diese Daten identifizierten einen zuvor unbekannten auf miRNA-basierenden Mechanismus der IL-6 Signalkaskade und tragen somit dazu bei, die komplexe Regulation der IL-6 Signaltransduktion besser zu verstehen.

Acknowledgments

I would like to thank Prof. Steffen Gay, Prof. Renate Gay and Prof. Rudolf Speich for giving me the opportunity to work in this exciting field and for sharing their great knowledge with me.

A very special thank you goes to my supervisor Dr. Lars Huber. His support, his optimism, his humor and his assistance were of essential importance for me to accomplish this thesis.

My partner Michelle Trenkmann was a great support for me during the whole period of my thesis, scientifically and privately, and I am very grateful to her. I also thank my parents and my sister for their help and advice.

Last but not least, I would like to say thank you to Dr. Astrid Jüngel, Dr. Caroline Ospelt, Emmanuel Karouzakis, Fabienne Niederer, Maria Comazzi, Benvinda Henriques, Peter Künzler, Ference Pataky, and all the members of the Center of Experimental Rheumatology in Zurich.

Contents

Introduction

1.1. Background

Pulmonary arterial hypertension (PAH) is a fatal disease characterized by the sustained increase in mean pulmonary artery pressure (> 25 mm Hg) that leads to exercise limitations, syncope, and, when left untreated, to right heart failure and death (Humbert *et al*., 2004a) With about 2-3 cases per million per year, PAH is defined as a rare disease that may be heritable, idiopathic (without identification of known risk factors) or associated with other disorders (e. g. collagen vascular diseases, portal hypertension, etc.) (Machado *et al*., 2009). However, by accounting all forms of pulmonary hypertension (PH) as provided in Table 1 (WHO classes of PH, Dana Point 2008), significant elevation of pulmonary vascular resistance represents a clinically important disease.

Table 1 Venice Clinical Classification of Pulmonary Hypertension (2003)

1. Pulmonary arterial hypertension (PAH)
1.1. Idiopathic (IPAH)
1.2. Familial (FPAH)
1.3. Associated with (APAH)
1.3.1. Collagen vascular disease
1.3.2. Congenital systemic-to-pulmonary shunts
1.3.3. Portal hypertension
1.3.4. HIV infection
1.3.5. Drugs and toxins
1.3.6. Other (thyroid disorders, glycogen storage disease, Gaucher disease, hereditary hemorrhagic telangiectasia, hemoglobinopathies, myeloproliferative disorders, splenectomy)
1.4. Associated with significant venous or capillary involvement
1.4.1. Pulmonary veno-occlusive disease (PVOD)
1.4.2. Pulmonary capillary hemangiomatosis (PCH)
1.5. Persistent pulmonary hypertension of the newborn

2. Pulmonary hypertension with left heart disease
2.1. Left-sided atrial or ventricular heart disease
2.2. Left-sided valvular heart disease

3. Pulmonary hypertension associated with lung diseases and/or hypoxemia
3.1. Chronic obstructive pulmonary disease
3.2. Interstitial lung disease
3.3. Sleep-disordered breathing
3.4. Alveolar hypoventilation disorders
3.5. Chronic exposure to high altitude
3.6. Developmental abnormalities

4. Pulmonary hypertension owing to chronic thrombotic and/or embolic disease
4.1. Thromboembolic obstruction of proximal pulmonary arteries
4.2. Thromboembolic obstruction of distal pulmonary arteries
4.3. Nonthrombotic pulmonary embolism (tumor, parasites, foreign material)

5. Miscellaneous

Sarcoidosis, histiocytosis X, lymphangiomatosis, compression of pulmonary vessels (adenopathy, tumor, fibrosing mediastinitis)

Table 1. Clinical classification of pulmonary hypertension (reproduced from: Humbert and McLaughlin, 2009)

Despite the fact that not all types of PH share a common etiology it is widely accepted that the observed increases in vascular resistance are caused by three factors: vasoconstriction, microthrombosis *in situ* and vascular remodeling which mainly affects small pulmonary arteries (Humbert *et al*., 2004b). The different forms of PH thus might share a final common pathway within the pathogenesis. The contraction of smooth muscle cells (SMCs) surrounding the small arteries is mainly associated with an increased expression of the vasoconstrictor endothelin-1 (*EDN1*) in the vasculature, whereas the release of the vasodilators nitric oxide (NO) and prostacyclins (e. g. prostaglandin I_2) from endothelial cells

(ECs) is reduced (Humbert *et al*., 2004b). On the other hand, narrowing of the arterial lumen can also be caused by coagulation of blood cells (microthrombosis) or by increased proliferation of ECs and SMCs leading to a thickened vessel wall (vascular remodeling, Figure 1).

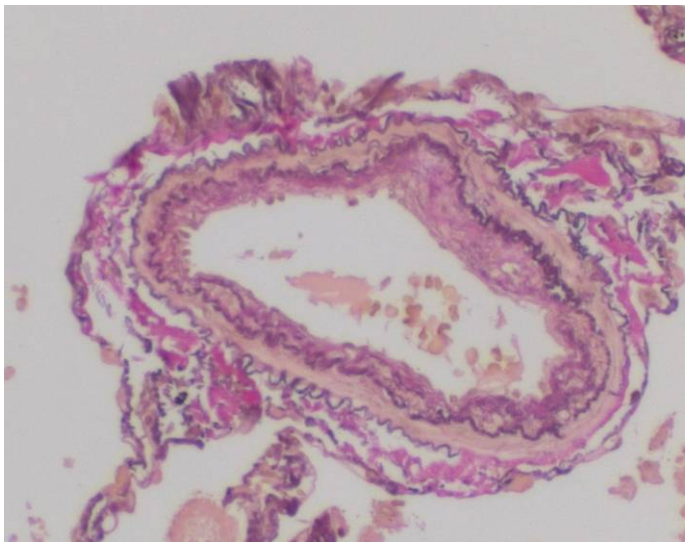

Figure 1. Artery from the lungs of a patient with PH
The intimal and the smooth muscle cell layer show a strong increase in size due to vascular remodeling and aberrant proliferation (reproduced from: Brock M and Huber LC, Rheuma-Nachrichten, Rheumaklinik und Insitut für Physikalische Medizin, UniversitätsSpital Zürich, Ausgabe Nr. 47, 2008)

The development of modern therapies significantly improved the life expectancy of patients suffering from P(A)H. Successfully employed strategies comprise the blocking of the endothelin receptors by an antagonist (such as *Bosentan*, Channick *et al.*, 2001) or the inhibition of the phopshodiesterase type 5 enzyme (e. g. by *Sildenafil*, Prasad *et al.*, 2000), which is responsible for the break-down of the vasodilator cyclic guanosine monophosphate (cGMP). Despite the improvement in life quality current therapies fail to cure PAH, which is probably due to the fact that they primarily target the imbalance between vasoconstrictors and vasodilators, whereas the process of vascular remodeling still continues. Therefore, in the last years several studies have focused on the process of vascular remodeling in order to understand the biological mechanism of this disorder and, hopefully, to reverse the once established disease.

In this regard, human genetic investigations shed new light on the pathogenesis of PAH by identifying bone morphogenetic protein receptor type II (*BMPR2)* as the gene responsible for approximately 70% of hereditary PAH and 20% of idiopathic PAH cases (Machado *et al*.,

2009). The gene *BMPR2* encodes a cell membrane receptor that, upon binding of its ligands (bone morphogenetic proteins, BMPs), heterodimerizes with members of the BMP receptor type I family, activates Smad transcription factors and finally controls the expression of multiple target genes (Davies and Morrell, 2008). The association of *BMPR2* gene defects and impaired vascular remodeling was supported by animal studies, which showed that *BMPR2* heterozygous mice had increased pulmonary arterial pressure and more muscularized vessels in the vasculature, a characteristic of vessel remodeling, when compared to wildtype (WT) animals (Beppu *et al*., 2004). Of interest, alterations in the surface expression of BMPR2 have also been described in non-genetic forms of PH (Atkinson *et al*., 2002) suggesting that dysregulation of the expression of BMPR2 is a common feature in the pathogenesis of PH.

Despite its important role in PH little is known about the biology of *BMPR2* gene regulation. Therefore, studies investigating the regulation of the expression of BMPR2 in the context of vascular remodeling and PH appear to be highly promising. The main focus of my PhD thesis was to elucidate mechanisms that control the expression of BMPR2 and to proof the physiological significance of these mechanisms in PH. Since the laboratory of Prof. Steffen Gay has a strong interest and expertise in the novel field of gene regulation by microRNAs (miRNAs), I focused my work on the regulation of BMPR2 by miRNAs.

In the first part of my PhD thesis, we identified a novel pathway involving the cytokine interleukin-6 (IL-6) and the polycistronic miRNA cluster miR-17/92 that tightly controls the expression of BMPR2. The physiological impact of this pathway was further proven in an *in vivo* model of PH (i. e. hypoxia-induced PH) and is presented as the second part of this thesis. Finally, shown in the last part of my thesis, we elucidated the interplay of IL-6 with miR-17/92 and its role in an acute inflammatory process, i.e. the acute-phase response.

1.2. BMPR2 in pulmonary hypertension

In hereditary PAH, germline mutations in the gene encoding *BMPR2* comprise a genetic hallmark of the disease (Rabinovitch, 2008). In the human body, BMPR2 is expressed in a tissue-specific manner with a prominent expression found on the surface of ECs and SMCs of the pulmonary arterial circulation (Atkinson *et al*., 2002). The gene *BMPR2* belongs to the transforming growth factor-β (TGF-β) superfamily and expresses a 70-80 kDa trans-membrane serine-threonine kinase receptor that facilities the binding of BMPs (Miyazono *et al*., 2010). BMPs, originally identified as factors that regulate the formation of bones and cartilage, exhibit a broad range of biological functions including the development of kidney, muscle, and neurons and vascular homeostasis (Kawabata *et al*., 1998). The receptor BMPR2

typically associates with the ligands BMP-2 and BMP-4, which are released from the endothelium, whereas the binding of other BMPs, mainly activins and myostatins, is achieved by BMPR2-related receptors (e. g. activin receptor type II, ActR2) (Miyazono *et al*., 2010).

For BMP signal transduction, the presence of both receptors, BMPR2 and BMPR1, is required. Binding of the ligand initiates the BMPR2-mediated transphosphorylation of the GS (glycine and serine-rich) domain of BMPR1. The phosphorylation of the GS domain strongly increases the serine-threonine kinase activity of BMPR1 that subsequently leads to the phosphorylation of associated R-Smad (receptor-regulated Smad) factors (typically Smad1, Smad5 and Smad8). Next, R-Smads interact with common-partner Smads (Co-Smads, typically Smad4 in the BMP-2 signaling cascade) to form an oligomeric protein complex. This oligomeric Smad complex then translocates to the nucleus, where it associates with a variety of transcription factors and coactivators which, eventually, leads to an altered transcriptional process of BMP target genes (Hardwick *et al*., 2008) (Figure 2).

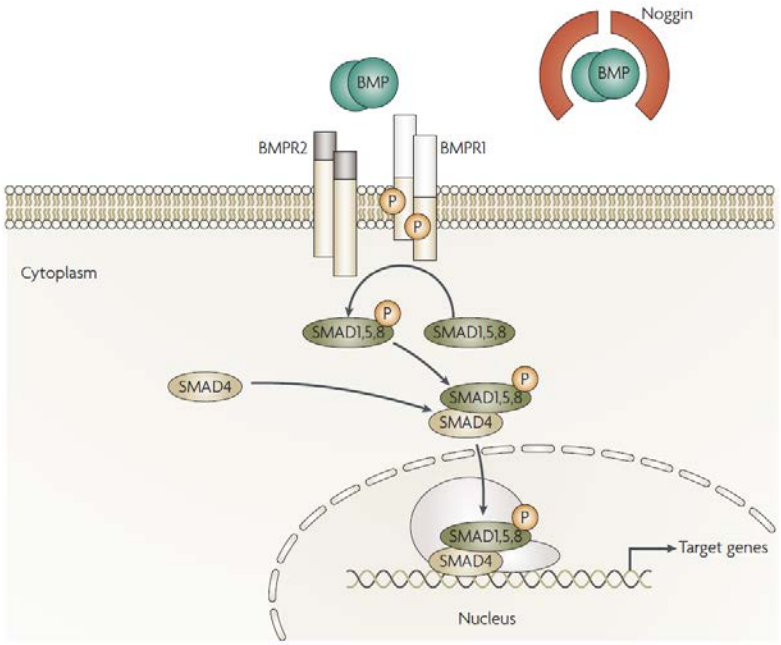

Figure 2. BMP signaling pathway

The schematic illustrates the activation of the BMP signaling cascade which is initiated by binding of BMPs to a heterodimeric receptor complex composed of the serine-threonine kinases BMPR1 and BMPR2. The BMPR2-mediated activation of BMPR1 triggers phosphorylation and activation of R-Smad transcription factors (Smad1, Smad5 and Smad8) which, guided by Co-Smads (Smad4), then shuttle to the nucleus to activate transcription of target genes. The BMP signaling can be inhibited by extracellular antagonists (e. g. noggin) that bind BMPs and thereby suppress the association with the receptor complex (reproduced from: Hardwick *et al*., 2008).

To achieve a finely balanced response to BMP stimulation, regulatory factors can interact at several levels of the pathway. For instance, the extracellular antagonist noggin binds BMP-4 with high affinity and thus abolishes the association of BMP-4 with its receptor (Zimmerman *et al*., 1996). Moreover, intracellular inhibitor-Smads (I-Smads, typically Smad6 or Smad7) prevent BMP-mediated signaling by decreasing the accessibility of R-Smads to BMPR1 (Miyazono *et al*., 2010). BMP signaling in the healthy lung endothelium plays an important role in the vascular maintenance by regulating the differentiation, proliferation, and apoptosis of ECs and SMCs (Davies and Morrell, 2008). The BMP-2 induced target gene family inhibitor of DNA binding (Id), for instance, controls differentiation and proliferation of human pulmonary arterial SMCs (Yang *et al*., 2010).

PH is characterized by a dysfunctional BMP and TGF-β signaling pathway. On the one hand, genetic studies identified frameshift or nonsense DNA mutations in the genes encoding the receptors for BMPs (*BMPR2*) and TGF-β (*TGFBR1* and *TGFBR2*) which cause a rapid degradation of the transcripts and thus loss of gene expression (Humbert *et al*., 2004a). On the other hand, reduced expression of BMPR2 and TGFBR2 were also found in patients with non-genetic (idiopathic) PAH (Atkinson *et al*., 2002; Yeager *et al*., 2001) and in animal models mimicking the disease (Takahashi *et al*., 2006; Morty *et al*., 2007). There is increasing evidence that the observed changes in the proliferation of SMCs which promote the vascular remodeling are a consequence of defective Smad signaling caused by the loss of BMPR2 expression (Yang *et al*., 2005). The therapeutical potential of BMPR2 to relieve the devastating condition of PH was tested in animal studies. In 2007, Reynolds and co-workers developed an adenoviral construct comprising the coding sequence of BMPR2 and specifically transferred the *BMPR2* gene to the lungs of rats (Reynolds *et al*., 2007). The delivery of the *BMPR2* gene successfully lowered the pulmonary hypertensive response to chronic hypoxia as compared to mock-infected controls. Moreover, by performing *in vitro* experiments, the authors found increased Smad signaling and reduced proliferation of BMPR2-transfected ephithelial cells. The authors concluded that restoration of BMPR2 expression by gene transfer therapy could be a promising tool for treatment of PH (Reynolds *et al*., 2007). In contrast to this study, McMurtry *et al*. demonstrated that the nebulized administration of an adenovirus containing the *BMPR2* gene failed to lower the pulmonary arterial pressure in the monocrotaline rat model for PH (McMurtry *et al*., 2007) which might be explained by differences in the methodology (gene delivery) and in the animal models used in both studies. However, more studies are needed to address the question whether gene therapy of BMPR2 protects or reverses the development of PH.

Restoration of vascular expression of BMPR2 remains an attractive approach for treatment of PH. In this regard, further characterization of the regulation of BMPR2 during the pathogenesis of PH is needed to better understand the mechanisms leading to reduced vascular expression of BMPR2. The identification of such mechanisms would allow the development of novel therapeutical approaches in order to restore functional levels of BMPR2 and to treat PH. Recent studies focusing on the regulation of BMPR2 in the development of PH have observed reduced protein but not mRNA levels of this surface receptor. Takahasi *et al*., for instance, described the expression of BMPR2 in pulmonary arteries of rats under normal conditions and after exposure to hypoxic conditions. Thereby, hypoxia was found to reduce the expression of BMPR2 on the protein levels, whereas the levels of the corresponding mRNA were not affected accordingly (Takahashi *et al*., 2006). Similarly, in the monocrotaline-induced rat model of PH, the expression of the BMPR2 protein was rapidly reduced without initial effects on the mRNA levels (Morty *et al*., 2007).

These findings suggest a post-transcriptional mechanism for the regulation of BMPR2, such as the involvement of miRNAs that bind to their target mRNAs by Watson-Crick-base pairing at distinct seed regions and, thus, alter mRNA stability or affect protein translation.

1.3. miRNAs in pulmonary hypertension

miRNAs comprise a novel class of short non-protein-coding RNA molecules that have emerged as novel important regulators of gene expression. Since the first description of miRNAs in the nematode *C. elegans* (Lee *et al*., 1993) miRNAs were found throughout the genomes of animals and plants, indicating the biological significance of these RNA-based regulators of gene expression. Up to date, more than one thousand miRNA sequences are identified in the human genome (source: www.mirbase.org; Griffiths-Jones *et al*., 2008) and it was suggested that up to one third of the human protein-coding genes is regulated by miRNAs through post-transcriptional mechanisms (Lewis *et al*., 2005). Compared to the accumulating knowledge about the function of miRNAs little is known about the biogenesis and the regulation of miRNAs processing. In general, the biogenesis of miRNAs is characterized by a sequence of four steps: (i) the RNA polymerase (Pol II or Pol III) - mediated transcription; (ii) the cleavage of the primary transcript by the RNAse III enzyme Drosha; (iii) the exportin-5 - facilitated transport of the precursor (pre) miRNA into the cytoplasm; and (iiii) the final processing of the stem-loop shaped - pre-miRNA into its mature length by Dicer (summarized in Figure 3, Winter *et al*., 2009)

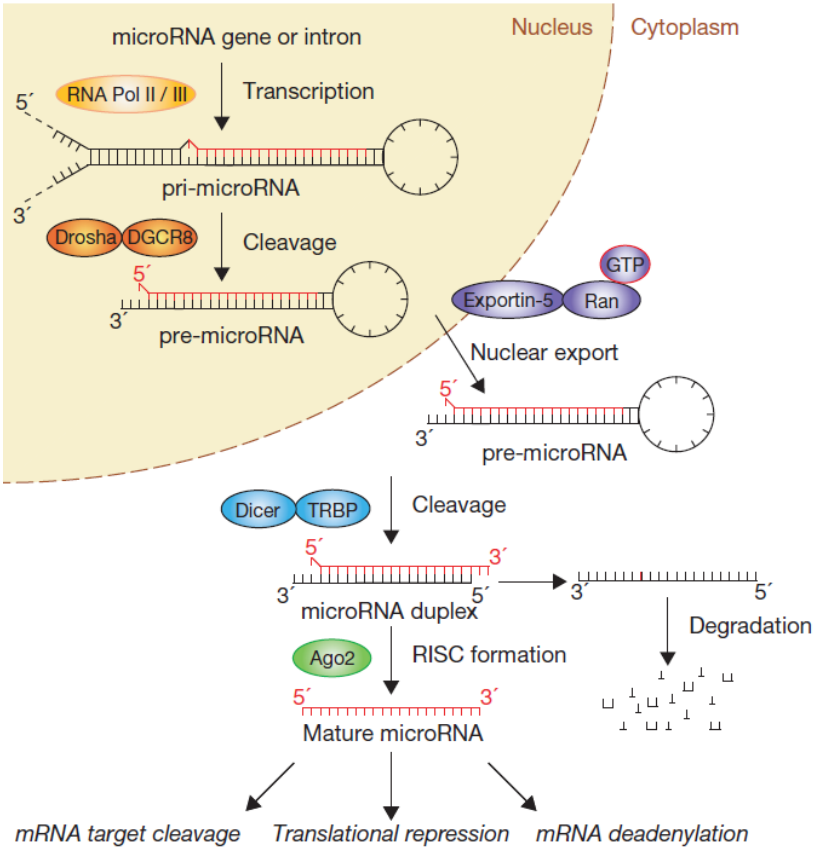

Figure 3. The pathway of miRNA processing

As a first step, the genomic region comprising the miRNA sequence is transcribed by the action of the RNA polymerase enzymes II or III and folds into a characteristic secondary structure. The primary, double-stranded miRNA (pri-miRNA) is then further processed by the RNase III enzyme Drosha leading to the formation of the pre-miRNA hairpin. Next, exportin-5 facilitates the transport into the cytoplasm where the RNase Dicer cleaves the pre-miRNA to its mature length. One strand of the mature miRNA, together with the RNA-binding protein Ago-2, is loaded onto RISC (RNA-induced silencing complex). The miRNA strand guides RISC to target mRNAs which are either cleaved, deadenylated or blocked in order to repress the translational process. (reproduced from: Winter *et al.*, 2009)

After the last processing step, one strand of the mature miRNA duplex is loaded onto the RNA-induced silencing complex (RISC) which then anneals to the miRNA-targeted mRNA to either cleave or block the translation of these targeted transcripts (Bartel, 2004). The complementarity of each miRNA to its targets decides which post-transcriptional modification is chosen: if there is a sufficient complementary the RISC is likely to catalyze the cleavage of the target mRNA; on the other hand, translational inhibition is characterized by an imperfect but still suitable pairing of miRNA and mRNA (Hutvagner and Zamore, 2002). Watson-Crick-base pairing occurs between the 5’ end of miRNA (known as the ‘seed’)

and a complementary region within the targeted mRNA, commonly located in the 3' untranslated region (UTR) (Lewis *et al*., 2003). Interestingly, miRNA-regulated genes tend to have longer 3'UTR, whereas house-keeping genes have shorter 3'UTRs, probably to escape from miRNA-mediated gene regulation (Stark *et al*., 2005). Because of the fact that the seed region of miRNA comprises only 7-8 nucleotides which theoretically could lead to the binding of hundreds of transcripts by only one single miRNA the identification of target genes remains challenging (Stark *et al*., 2003). In the last years new approaches have been developed that allow an efficient and fast identification of potential miRNA targets. Beside the usage of computer-based miRNA target prediction programs proteomic studies showed great potential in identifying miRNA targets and, moreover, demonstrated that one miRNA can influence the expression of hundreds genes (Selbach *et al*., 2008).

miRNAs have been associated with various biological processes including development, differentiation and malignancy (Mendell, 2005). The role of miRNAs in vascular biology was first proven by miRNA-depletion studies showing that miRNAs are required for normal blood vessel development, vascular integrity and vasoconstriction (Sen *et al*., 2009). By screening the miRNA profile during the development of PH in the lungs of chronic hypoxic or monocrotaline-treated rats Caruso and colleagues reported dynamic changes in the expression of Dicer and distinct miRNAs (Caruso *et al*., 2010). In both models used the authors found reduced expression of let-7f, miR-30c and miR-22 whereas the levels of miR-322, miR-451 and miRNAs derived from the polycistronic miRNA cluster miR-17/92, namely miR-17 and miR-20a, were upregulated indicating a potential role of these miRNAs in the pathogenesis of PH. However, Caruso *et al.* did not investigate the functional role of these miRNAs (Caruso *et al*., 2010), therefore it remains speculative whether these identified miRNAs are important factors in the pathogenesis of PH. Nonetheless, a recent publication provided evidence that miRNAs are critical for the development of PAH. Courboulin *et al.* showed that miR-204 is significantly downregulated in human PAH and in animal models for PH thereby contributing to the anti-apoptotic phenotypes of SMCs (Courboulin *et al*., 2011). Moreover, this study addressed two important points: on the one hand, Courboulin and colleagues demonstrated that miR-204 can be used as a novel biomarker in buffy coat samples of patients with PAH and, on the other hand, that administration of synthetic miR-204 relieves the severity of monocrotaline-induced PH in rats. Consequently, the authors concluded that miRNAs play a role in the pathogenesis of PAH and that therapies altering the expression of miRNAs *in vivo* might represent a novel tool for the treatment of this disease (Courboulin *et al*., 2011).

Decreased BMP signaling represents a common feature of PAH (Morty *et al*., 2007). In this regard, novel data illustrated the interplay of BMP signaling and miRNAs. Davis and colleagues, for instance, demonstrated that the induction of a contractile phenotype of HPASMC is mediated by miR-21 which in turn is strongly upregulated by TGF-β and BMP stimulation (Davis *et al*., 2008). In addition, further studies provided evidence that the BMP-related TGF-β signaling can be regulated by miRNAs derived from miR-17/92. Dews *et al.* reported that over expression of miR-17/92 results in reduced gene activation by TGF-β (Dews *et al*., 2010). In more detail, the authors demonstrated a specific knock down of two important components of the TGF-β signaling cascade, namely TGFBR2 and Smad4, by miR-17/92 which finally blunts TGF-β signaling (Dews *et al*., 2010).

Based on the findings that (i) the expression of miR-17/92 is increased in experimental models of PH (Caruso *et al*., 2010) and that (ii) miR-17/92 represses TGF-β signaling (Dews *et al*., 2010) one can postulate an important contribution of miR-17/92 to the altered activity of BMP signaling observed during the development of PH. Interestingly, because of its polycistronic organization (miR-17/92 encodes in total six different miRNAs: miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92) miR-17/92 is associated with many different biological processes including heart and lung development and postnatal neovascularization (Bonauer and Dimmeler, 2009). On the molecular level, several independent lines of evidences reported that miR-17/92 tightly controls the proliferation and survival of several cell lines by targeting the anti-apoptotic gene *Bim* (Bcl-2-interacting mediator of cell death; Xiao *et al*., 2008) and the cell growth inhibitor *p21* (Fontana *et al*., 2008).

Based on these findings, the role of miR-17/92 in the development of PH appeared to be promising and was therefore selected as a main subject of my PhD thesis. On the one hand, we showed that BMPR2, a key molecule in the pathogenesis of PH (Morrell, 2010), is targeted by miR-17/92. On the other hand we provided evidence that the expression of miR-17/92 itself is regulated by the cytokine IL-6 and thereby offered a novel link between inflammation and the pathogenesis of PH.

1.4. Inflammation and Pulmonary Hypertension

Inflammatory mechanisms appear to significantly contribute to some classes of PH including PAH associated with connective tissue diseases and human immunodeficiency virus infection as well as monocrotaline-induced PH in rats (Humbert *et al*., 2004a). Evidence for an impaired immunological homoeostasis were also found in some patients suffering from idiopathic PAH suggesting a potential role for inflammation in the development of this disease. In severe PAH, for instance, infiltrating lymphocytes and macrophages in plexiform lesions have been described (Tuder *et al*., 1994) as well as enhanced expression levels of the chemokines RANTES (regulated upon activation normal T cell expressed and secreted; Dorfmuller *et al*., 2002) and fractalkine (Balabanian *et al*., 2002).

With regard to PH, the pleiotropic cytokine IL-6, which was found to be associated with a large number of disorders including pulmonary vascular diseases (Neurath and Finotto, 2011), and its downstream signaling factors such as STAT3 (Signal transducer and activator of transcription 3) are of special interest.

The IL-6 pathway has been intensively studied in the last years and is well described. Briefly, upon binding to its cell surface receptor IL-6 activates transcription factors that in turn enhance the expression of IL-6 target genes including pro-proliferative factors (e. g. c-Myc) and anti-apoptotic molecules (e. g. Bcl-2) (Steiner *et al*., 2009). In general, the binding of IL-6 initiates the heterodimerization of the IL-6 receptor, gp80, and the IL-6 signal transducer, gp130 (Montero-Julian, 2001). The receptor assembly triggers three sequential phosphorylation events that are carried out by the receptor-associated Janus kinases (JAKs): (1) transphosphorylation and activation of the JAKs; (2) tyrosine phosphorylation of the receptor tails; and (3) phosphorylation of the transcription factor STAT3 at tyrosine residue 705 (Heinrich *et al*., 1998a). Phosphorylated and activated STAT3 homodimerizes and shuttles to the nucleus where it activates the transcription of IL-6 target genes (Seidel *et al*., 1995; the IL-6 / STAT3 pathway is summarized in Figure 4).

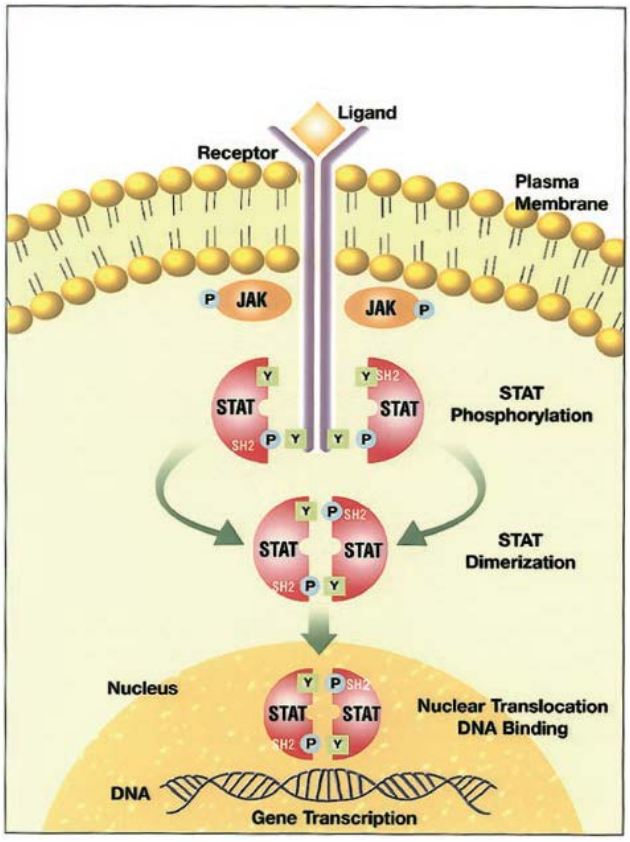

Figure 4. IL-6 / STAT3 signal transduction pathway
Upon binding of IL-6 to its receptor the phosphorylation of the receptor-associated JAKs is initiated. Subsequent phosphorylation of the receptor tails enables the binding and the activation of the transcription factor STAT3, which then dimerizes and shuttles to the nucleus. Phosphorylated STAT3 binds to IL-6 responsive elements located in gene promoters in order to promote the transcription of these genes (reproduced from: Benekli *et al*., 2003)

Several negative feedback loops antagonize the activation of STAT3 to avoid persistent downstream signaling. A well characterized feedback pathway includes the blocking of the phosphorylation of STAT proteins by the action of the cytokine-inducible protein family of SOCS (Suppressors of Cytokine Signaling) (Krebs and Hilton, 2001). The transcriptional activity of STAT3 can further be inhibited either by the blocking of the DNA binding domain of STAT3 mediated by PIAS3 (Protein Inhibitor of Activated STAT 3; Chung *et al*., 1997) or by the dephosporylation of activated STAT3 achieved by cytoplasmatic phosphatases (e. g. SHP-1 or SHP-2; Benekli *et al*., 2003)
Intriguingly, patients with PAH were found to have higher serum levels of the proinflammatory cytokines IL-1 and IL-6 as compared to healthy controls (Humbert *et al*., 1995) and, moreover, serum levels of IL-6 can be used to predict survival of PAH patients (Soon *et al*., 2010). The impact of IL-6 on the pathogenesis of PH has been intensively

investigated in recent years. Two studies showed that ectopic administration of IL-6 is capable of inducing a mild form of PH. Golembeski and colleagues demonstrated that subcutaneous injections of IL-6 caused an increase of right ventricular pressure in rats under normoxic conditions (Golembeski *et al*., 2005). Consequently, as shown by Steiner *et al.*, lung-specific overexpression of IL-6 in transgenic mice was found to enhance the vascular resistance and, furthermore, to increase the muscularization of the pulmonary arteries, a characteristic feature of PAH (Steiner *et al*., 2009). Conversely, by performing loss-of-function experiments Savale *et al*. showed that IL-6 knock-out mice had reduced right ventricular pressure and decreased media thickness of pulmonary vessels after exposure to chronic hypoxia as compared to wildtype mice (Savale *et al*., 2009). These findings highlighted a pivotal role of IL-6 in the development of PH and, moreover, introduced two potential novel experimental models with high pathogenetic relevance to the human disease. Whether IL-6 also plays a role in human PH associated with chronic hypoxia (e.g. patients with chronic obstructive pulmonary disease, COPD) and whether IL-6 is upregulated in these conditions (for example through hypoxia-responsive elements), however, remains elusive so far. It will be also interesting whether the known therapies targeting the IL-6 pathway such as Actemra®, an antibody binding to the receptor of IL-6 (Nishimoto and Kishimoto, 2006), is of benefit for patients with PH.

Moreover, a dysregulation of STAT3 signaling indicated by persistent activation of STAT3 was reported in a study published by Masri and colleagues (Masri *et al*., 2007). By investigating the phosphorylation state of STAT3 in ECs derived from characteristic plexiform lesions within pulmonary arteries of patients with idiopathic PAH the authors found increased activation of STAT3 when compared to healthy donors. In addition, the enhanced STAT3 activity was accompanied by upregulated expression of pro-proliferative and anti-apoptotic molecules (Mcl-1 and Bcl-2; Masri *et al*., 2007). The authors concluded that the observed phenotype of enhanced cell growth and survival of ECs is strongly associated with an abnormal and persistent activation of STAT3 (Masri *et al*., 2007). The fact that increased phosphorylation of STAT3 was mainly detected in plexiform lesions of PAH patients (Masri *et al*., 2007) further indicates a potential link between vascular remodeling and activation of STAT3.

In some cell types it has been shown that BMP-2 signaling induces apoptosis mainly mediated by an immediate inactivation of STAT3 (Kawamura *et al*., 2000). In this regard, one can speculate that the loss of BMPR2 as observed in P(A)H leads to increased activation of

STAT3 accompanied by enhanced proliferation of endothelial and smooth muscle cells and, ultimately, to elevated pulmonary arterial pressure (Mathew, 2010).

1.5. Objectives

The regulation of BMPR2 by microRNAs

The pathogenesis of PH is characterized by vascular remodeling and vasoconstriction (Humbert *et al*., 2004b). In hereditary PAH, mutations in the *BMPR2* gene leading to loss of expression of BMPR2 comprise a genetic hallmark of the disease (Machado *et al*., 2009). BMPR2 is a cell surface receptor expressed on endothelial and vascular smooth muscle cells and mediates the binding of BMP ligands to the cell surface. BMPs were shown to inhibit proliferation of smooth muscle cells (Wong *et al*., 2003), a process which requires the presence of BMPR2 (Yu *et al*., 2008). Thus, it was suggested that the reduced expression of BMPR2 might lead to significant alterations in the signaling cascade and, ultimately, to vascular remodeling. Of interest, decreased expression of BMPR2 has also been found in non-genetic forms of PH (Atkinson *et al*., 2002). The intracellular mechanisms leading to this downregulation, however, have not been unraveled so far.

Studies focusing on the role of BMPR2 in the development of PH have observed reduced protein levels of this receptor, whereas the levels of the corresponding mRNA were not affected accordingly (Takahashi *et al*., 2006; Morty *et al*., 2007). These findings suggest that the expression of BMPR2 is regulated by post-transcriptional mechanisms. Since miRNAs have emerged as important post-transcriptional regulators of gene expression that alter the stability of target mRNAs or affect protein translation, we hypothesized an involvement of miRNAs in the regulation of BMPR2.

In the first part of this PhD thesis, we raised the question whether miRNAs could control the expression of BMPR2. Using a miRNA target prediction program (i.e. TargetScan; Lewis *et al*., 2005) we identified the miRNA cluster miR-17/92 as a potential modulator of BMPR2 expression. Therefore, in the first study, we addressed the following issues: (i) the role of miR-17/92 in the post-transcriptional regulation of BMPR2 expression; (ii) the effects of inflammatory cytokines (i. e. IL-6) on the expression of miR-17/92; and, (iii) the role of the transcription factor STAT3 as a master link between IL-6 and the expression of BMPR2. Experiments to study the functional impact of miR-17/92 on BMPR2 were carried out in human embryonic kidney (HEK)293 cells that were shown to express functional levels of

BMPR2 (Ramos *et al*., 2006), whereas IL-6 stimulation experiments were performed in human pulmonary arterial endothelial cells (HPAEC).

Silencing of miRNAs in an *in vivo* model of PH

We proposed that miRNAs regulate the expression of BMPR2 and, in the first study, we identified a pathway involving the IL-6-inducible transcription factor STAT3 and a miR-17/92 derived miRNA, miR-20a (Brock *et al*., 2009). Our hypothesis of a miR-20a-mediated downregulation of BMPR2 in PH was further supported by Caruso *et al.* showing that miRNAs encoded by miR-17/92 are overexpressed during the development of PH in the lungs of chronic hypoxic or monocrotaline-treated rats (Caruso *et al*., 2010). Our pathway thus might represent a unifying mechanism in the pathogenesis of several forms of PH. Consequently, miR-17/92 appeared to be an interesting target for further investigations. Regarding the pathogenesis of PH and therapies involving *BMPR2* gene delivery (Reynolds *et al*., 2007), our model offered the potential to increase levels of endogenously expressed BMPR2 by antagonizing miR-20a.

The efficient and non-toxic inhibition of miRNAs *in vivo* is subject of intensive research considering that such methods could be used as novel therapeutic approaches to target miRNA expression in human disease. In this regard, antagomiRs emerged as promising and appropriate tools (Krutzfeldt *et al*., 2005). AntagomiRs are small anti-sense RNA molecules directed against miRNA sequences that are further modified in order to improve their stability (methylation of the RNA backbone) and delivery (cholesterol conjugation) (Krutzfeldt *et al*., 2005).

In the second part of my PhD thesis, we investigated feasibility and functional readout of specific antagonization of miR-20a by using antagomiRs in a mouse model of hypoxia-induced pulmonary hypertension. The following issues were addressed: (i) the efficiency of miR-20a knock down by intraperitoneal injections of antagomiR-20a; (ii) the functional consequence of miR-20a knock down on vascular remodeling and right heart hypertrophy; and, (iii) the potential of antagomiR-20a to restore functional expression levels of BMPR2 in the lungs of chronic hypoxic mice.

miR-17/92 and IL-6 signaling

We provided evidence that the cytokine IL-6 and its downstream signaling factors (i.e. STAT3), respectively, regulate the expression of miR-17/92 in pulmonary endothelial cells. In addition, we identified a palindromic TT – AA motif with a spacing of four base pairs in the promoter of miR-17/92, which indicates the binding of the IL-6-inducible transcription factor STAT3. These findings emphasize a potential link between miR-17/92 and inflammatory processes (Brock *et al*., 2009). Since some IL-6-induced target genes contribute to feedback loops of IL-6 signaling (e. g. the SOCS genes; Krebs and Hilton, 2001) we hypothesized that miR-17/92 might represent a novel player within this signal transduction cascade.

The hepatic acute-phase response, which represents the systemic reaction to acute inflammation and tissue damage, is well characterized and represents one of the most important examples of IL-6 signaling (Heinrich *et al*., 1998b). Since the IL-6-induced acute-phase response in human hepatoma (HepG2) cells provide an established model for the IL-6 signaling pathway (Hou *et al*., 2007), we used these cells to investigate the role of miR-17/92 in the IL-6 signaling cascade

In this part of my PhD thesis, we addressed the following issues: (i) the role of miR-17/92 in the IL-6 signaling cascade and, in more detail, the impact on the expression of IL-6 downstream target genes such as the acute-phase genes (e. g. fibrinogen and haptoglobin); (ii) the identification of novel miR-17/92 targets within the IL-6 signaling cascade; and, (iii) the effect of overexpression of miR-17/92 on the phosphorylation and activation of STAT3.

2. Interleukin-6 modulates the expression of the bone morphogenetic protein receptor type II through a novel STAT3-microRNA cluster 17/92 pathway

Brock M, Trenkmann M, Gay RE, Michel BA, Gay S, Fischler M, Ulrich S, Speich R, Huber LC. Interleukin-6 modulates the expression of the bone morphogenic protein receptor type II through a novel STAT3-microRNA cluster 17/92 pathway. *Circ Res* 2009;**104**(10):1184-91.

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Circulation Research 2009, 104:1184-1191: originally published online April 23, 2009
doi: 10.1161/CIRCRESAHA.109.197491
Circulation Research is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Matthias Brock, Michelle Trenkmann, Renate E. Gay, Beat A. Michel, Steffen Gay, Manuel Fischler, Silvia Ulrich, Rudolf Speich, Lars C. Huber

Abstract—Dysregulated expression of bone morphogenetic protein receptor type II (BMPR2) is a pathogenetic hallmark of pulmonary hypertension. Downregulation of BMPR2 protein but not mRNA has been observed in multiple animal models mimicking the disease, indicating a posttranscriptional mechanism of regulation. Because microRNAs (miRNAs) regulate gene expression mainly through inhibition of target gene translation, we hypothesized that miRNAs may play a role in the modulation of BMPR2. Performing a computational algorithm on the BMPR2 gene, several miRNAs encoded by the miRNA cluster 17/92 (miR-17/92) were retrieved as potential regulators. Ectopic overexpression of miR-17/92 resulted in a strong reduction of the BMPR2 protein, and a reporter gene system showed that BMPR2 is directly targeted by miR-17-5p and miR-20a. By stimulation experiments, we found that the miR-17/92 cluster is modulated by interleukin (IL)-6, a cytokine involved in the pathogenesis of pulmonary hypertension. Because IL-6 signaling is mainly mediated by STAT3 (signal transducer and activator of transcription 3), the expression of STAT3 was knocked down by small interfering RNA, which abolished the IL-6–mediated expression of miR-17/92. Consistent with these data, we found a highly conserved STAT3-binding site in the promoter region of the miR-17/92 gene (C13orf25). Promoter studies confirmed that IL-6 enhances transcription of C13orf25 through this distinct region. Finally, we showed that persistent activation of STAT3 leads to repressed protein expression of BMPR2. Taken together, we describe here a novel STAT3–miR-17/92—BMPR2 pathway, thus providing a mechanistic explanation for the loss of BMPR2 in the development of pulmonary hypertension. (***Circ Res*****. 2009;104:1184-1191.)**

Key Words: pulmonary hypertension ■ BMPR2 ■ miR-17/92 ■ interleukin-6 ■ STAT3

Pulmonary hypertension is a devastating condition defined by the sustained elevation of pulmonary vascular resistance that leads rapidly to right heart failure and death when left untreated.[1] The pathogenesis of pulmonary hypertension is characterized by vascular remodeling and vasoconstriction.[2] Many chemotactic and inflammatory factors have been associated with these vascular changes including interleukin (IL)-6 and transforming growth factor (TGF)β.[3–5] In familial pulmonary arterial hypertension, germline mutations in the gene encoding the type II receptor of the bone morphogenetic protein (BMPR2) comprise a genetic hallmark of the disease.[6] BMPR2 is a surface protein receptor that belongs to the transforming growth factor (TGF)β family. Its expression on endothelial and vascular smooth muscle cells mediates binding of bone morphogenetic proteins (BMPs) that have been identified as inhibitors of vascular smooth muscle cell proliferation while inducing cell death.[7] Thus, it was suggested that the downregulation of BMPR2 might lead to significant alterations in these signaling cascades and, ultimately, to remodeling of the pulmonary vascular bed.[8] Of interest, alterations in the surface expression of BMPR2 have also been described in nongenetic forms of pulmonary hypertension.[9] In addition, loss of BMPR2 has been observed in several animal models mimicking the disease.[8,10] The intracellular mechanisms leading to this downregulation however are yet to be elucidated. Studies focusing on the role of BMPR2 in the development of pulmonary arterial hypertension have observed reduced protein level of this surface receptor. Takahashi et al, for example, described the expression of BMPR2 in pulmonary arteries of rats under normal conditions and after exposure to hypoxic conditions. Consequently, hypoxia was found to reduce the expression of BMPR2 on the protein levels, whereas the levels of the corresponding mRNA were not affected adequately.[8] Similarly, in the monocrotaline-induced rat model of pulmonary hypertension, the expression of the BMPR2 protein was rapidly reduced without initial effects on the mRNA levels.[10]

Original received January 8, 2009; resubmission received March 19, 2009; revised resubmission received April 14, 2009; accepted April 15, 2009.

From the Center for Experimental Rheumatology and Zurich Center for Integrative Human Physiology (M.B., M.T., R.E.G., B.A.M., S.G., L.C.H.), University of Zurich, Switzerland; and Working Group for Pulmonary Hypertension (M.B., M.F., S.U., R.S., L.C.H.), Department for Internal Medicine, University Hospital Zurich, Switzerland.

Correspondence to Lars C. Huber, MD, Department of Internal Medicine, University Hospital Zurich, Rämistrasse 100, Zurich 8091, Switzerland. E-mail lars.huber@usz.ch

Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/CIRCRESAHA.109.197491**

These findings suggest a posttranscriptional mechanism, such as the involvement of microRNAs (miRNAs) that bind to their target mRNAs by Watson–Crick base pairing at distinct seed regions and, thus, alter mRNA stability or affect protein translation.

An accumulating body of evidence suggests that up to one-third of the human genome is regulated by miRNAs through posttranscriptional mechanisms.[11] Consequently, miRNAs have been associated with various cellular processes including cell death, differentiation, and proliferation.[12] Based on computational algorithms (ie, TargetScan) and the fact that surface protein receptors such as the TGFβ-receptor type II (TGFβR2) have already been shown to be regulated by miRNAs,[13] we identified the miRNA cluster 17/92 as potential modulator of BMPR2 expression. In the present in vitro study, we used human pulmonary arterial endothelial cells (HPAECs), hepatocellular carcinoma (HepG2) cells, and human embryonic kidney (HEK)293 cells to address the following issues: (1) the role of microRNA (miR)-17/92 in the posttranscriptional regulation of BMPR2 expression; (2) the effects of inflammatory cytokines and growth factors on the expression of miR-17/92; and (3) the role of the transcription factor STAT3 (signal transducer and activator of transcription 3) as the master link between IL-6 and the modulation of BMPR2. Our data reveal for the first time a potential molecular mechanism explaining the downregulation of BMPR2 in the development of pulmonary arterial hypertension.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Cell Culture

For cell culture, human embryonic kidney (HEK)293 cells, and human hepatocellular carcinoma (HepG2) cells were used. HPAECs were purchased from Cascade Biologics. All growth factors and stimulation agents (recombinant human IL-6, vascular endothelial growth factor [VEGF], platelet-derived growth factor [PDGF]) were purchased from R&D Systems.

Plasmid Construction

For overexpression of the miR-17/92 cluster, genomic DNA encoding miR-17/92 was amplified and cloned into pcDNA3.1$^+$ between the *Hind*III and *Eco*RI restriction sites as previously described.[14] Cells were transfected using Lipofectamine 2000.

Real-Time RT-PCR Analysis

Total RNA was extracted using the RNeasy kit and quantification of specific RNA transcripts was performed by SYBR Green real-time PCRs using the ABI Prism 7700 Sequence Detection System.

Quantification of Mature miR-20a

Total RNA was extracted using the mirVana miRNA Isolation Kit. Mature miR-20a was detected by stem–loop reverse transcription, followed by SYBR Green real-time PCR[15] and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase.

Western Blot Analysis

The following antibodies were used for Western blot: anti-human BMPR2, anti-human STAT3, anti-human phospho-STAT3, and anti–α-tubulin. Evaluation of the expression of specific proteins was performed by the Alpha Imager Software system via pixel quantification of the electronic image.

Reporter Gene Assay

A 1554-bp fragment of the 3′ untranslated region (3′UTR) of BMPR2 was amplified out of genomic DNA. The PCR product was *Xba*I digested and cloned into the *Xba*I restriction site of the pGL3 control vector. As negative control, the antisense construct was used according to Kuhn et al.[16] HEK293 cells were transfected with the pGL3 control 3′UTR of BMPR2 "sense" or "antisense" construct. A vector encoding for the miR-17/92 cluster (pcDNA miR-17/92), and a vector for normalization (pRL-SV40) were added. For inhibition of endogenous miRNAs, a similar protocol was applied with the use of anti-miRs. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System, and the values obtained were normalized to the activity of Renilla luciferase.

Nuclear Transfection

The endogenous expression of the human STAT3 gene was knocked down by validated small interfering (si)RNA for STAT3. Nuclear transfection of HPAECs was achieved by using the nucleofection kit HMVEC-L from Amaxa.

Statistics

For statistical analysis, GraphPad Prism Software was used.

Results

Computational Analysis Reveals BMPR2 As a Target of the miR-17/92 Cluster

Data from previous studies on the protein expression of BMPR2 in animal models of pulmonary hypertension suggested a posttranscriptional mechanism of regulation, possibly involving miRNAs. We thus performed a screening based on a computational algorithm (TargetScan, Whitehead Institute for Biomedical Research, www.targetscan.org) to identify distinct seed regions of miRNAs in the 3′UTR of the BMPR2 gene. To improve prediction rates the focus was put only on highly conserved seed regions.[17] TargetScan retrieved several miRNAs for BMPR2, including miR-17-5p, miR-19a, miR-19b, miR-20a, and miR-92. Of interest, these miRNAs are encoded by one single miRNA cluster, miR-17/92 located at the chromosome 13q31 in the 3rd intron of the C13orf25 gene.[18]

Overexpression of miR-17/92 Reduces the Expression of BMPR2

To verify the predicted miRNAs from the computational analysis on a functional basis, the entire cluster was cloned into a mammalian expression vector under the control of the SV40 promoter. Successful overexpression in HEK293 cells was confirmed by quantification of one representative mature miRNA derived from miR-17/92 (miR-20a) 72 hours after transfection. As shown in Figure 1A, the expression of miR-20a was significantly increased by 2.71±0.45-fold (P=0.005) as compared with the transfection with the empty vector (mock). The overexpression of the miR-17/92 cluster resulted in a significant decrease of BMPR2 on the protein level as analyzed by Western blot (0.66±0.06 fold; P=0.002; Figure 1B). The time-dependent downregulation is shown in Figure I in the online data supplement. The corresponding levels of mRNA were also significantly reduced (0.89±0.06 fold; P=0.03; Figure 1C). However, the changes observed on

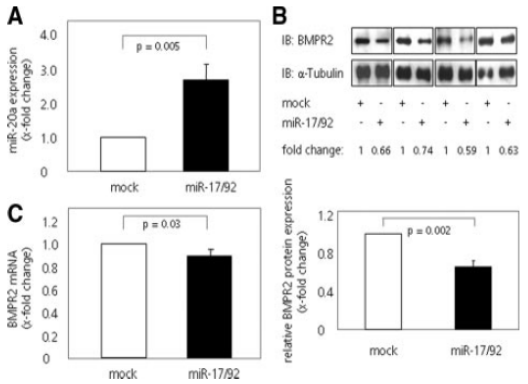

Figure 1. Overexpression of miR-17/92 in HEK293 cells. HEK293 cells were transfected with the pcDNA miR-17/92 vector, and mRNA and proteins were collected 72 hours later. A, Quantification of miR-20a showed a significant upregulation by 2.71±0.45-fold as compared with mock transfection. B, Transient overexpression of the miR-17/92 cluster led to a 40% decrease of BMPR2 protein, as analyzed by Western blot. C, Levels of BMPR2 mRNA were slightly reduced (0.89±0.06 fold). Data are shown as means±SD derived from 4 independent experiments.

the mRNA level were much less impressive than the protein changes, indicating that the miRNA cluster represses the translation process of BMPR2. The modest downregulation of mRNA levels, on the other hand, is a known phenomenon reflecting the interactive behavior of miRNAs and mRNA.[19]

The Reduction of BMPR2 Is Mediated Directly by the Action of miR-17-5p and miR-20a

In a next step, we addressed the question whether the observed reduction of BMPR2 protein is directly miRNA-driven. A part of the 3′UTR of BMPR2 (35 to 1589 bp) was cloned into the pGL3 control vector creating a luciferase reporter system with respective binding sites for the miRNAs 17-5p, -19a, -19b, -20a, and -92 (Online Figure II). The antisense construct was generated and used as negative control.[16] Luciferase activity was assessed, and data were normalized to the Renilla luciferase activity. Cotransfection of the pGL3 3′UTR of BMPR2 sense construct, and the miR-17/92–overexpressing vector yielded a lower relative luciferase activity as compared with mock when transfected into HEK293 cells (0.76±0.12 fold; Figure 2A). The antisense construct was not affected by overexpression of the miR-17/92 cluster (1.06±0.07 fold). These findings imply a direct interaction between the 3′UTR of BMPR2 and the miRNAs derived from the miR-17/92 cluster. To identify the impact of the distinct miRNAs, each endogenous miRNA from the cluster was inhibited by the use of antisense RNA molecules (anti-miRs). As shown in Figure 2B, these blocking experiments revealed a significantly higher relative luciferase activity for anti–miR-17-5p (1.31±0.21 fold; P=0.03) and anti–miR-20a (1.52±0.24 fold; P=0.008), indicating a pivotal role of these 2 miRNAs in the interaction with the BMPR2.

The Expression of miR-17/92 Correlates With the Activity of STAT3

A growing body of evidence suggests that the intracellular level of some miRNAs is regulated, at least in part, by the action of cytokines[20] and growth factors.[21] Because these factors have also been implicated in the pathogenesis of pulmonary hypertension,[4] we stimulated HPAECs with IL-6, VEGF, and PDGF and quantified the expression of the preliminary transcript of miR-17/92 (C13orf25).

Figure 3A illustrates a significant upregulation of C13orf25 mRNA 30 minutes and 1 hour after the stimulation of HPAECs with IL-6 (1.98±0.19 fold; P=0.0018 and 1.87±0.33 fold; P=0.045 respectively) and VEGF (1.96±0.36 fold; P=0.045 and 1.58±0.03 fold; P=0.001 respectively) as compared with unstimulated control cells. 4 hours after stimulation, the mRNA levels reached baseline expression. The stimulation with PDGF did not affect the mRNA levels of C13orf25, probably because of the fact that HPAECs lack the receptor for PDGF (data not shown).

It was reported from previous experiments in endothelial cells, that IL-6 and VEGF share common signaling mechanisms including the STAT3 pathway.[22,23] STAT3 is a major transcription factor, which is shuttled from the cytoplasm into the nucleus on activation by tyrosine phosphorylation of residue 705. When HPAECs were stimulated and analyzed by Western blotting, a strong phosphorylation of STAT3 could be observed for IL-6 after 30 minutes, and, to a lesser extent, after 1 hour. However, no phosphorylation could be detected after the addition of VEGF (Figure 3B). With respect to the data presented in Figure 3A, a correlation between activation of STAT3 and induction of the expression of C13orf25 can be postulated. According to this hypothesis, we performed siRNA experiments to knockdown the endogenous STAT3

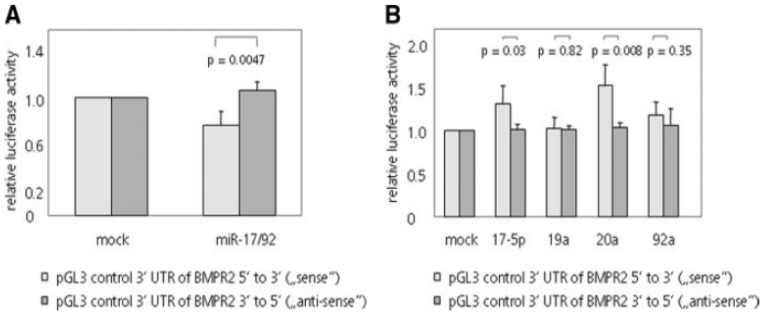

Figure 2. Reporter gene studies on the interaction between 3′UTR of BMPR2 and the miR-17/92 cluster in HEK293 cells. A, Cotransfection of the pGL3 3′UTR of BMPR2 sense construct and the miR-17/92–overexpressing vector resulted in a significantly lower relative luciferase activity as compared with the antisense construct (P=0.0047). B, Blocking experiments with the use of antisense RNA molecules (anti-miRs) directed against each miRNA revealed a significantly higher relative luciferase activity for anti–miR-17-5p and anti–miR-20a. Data are shown as means±SD derived from 4 independent experiments.

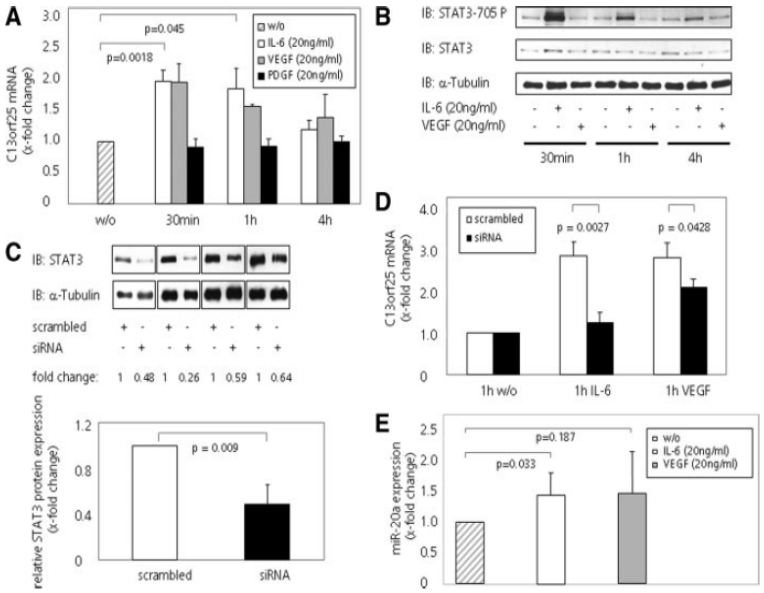

Figure 3. Correlation between the expression of miR-17/92 and the activity of STAT3 in HPAECs. A, Quantification of the preliminary transcript of miR-17/92 (C13orf25). Stimulation of HPAECs with IL-6 and VEGF but not PDGF significantly increased the expression of C13orf25 mRNA. B, Phosphorylation of STAT3 at tyrosine residue 705 could be observed for IL-6 in a time-dependent manner, whereas no effect was seen for the stimulation with VEGF. C, siRNA-mediated knockdown reduced the expression of STAT3 by 50% on the protein level. D, The induction of C13orf25 mRNA by IL-6 was almost completely abolished by the functional knockdown of STAT3 as compared with stimulated scrambled control cells (P=0.0027). The effect observed by stimulation with VEGF was less strong but still reached statistical significance (P=0.0428). E, Stimulation of HPAECs for 24 hours revealed significant upregulation of miR-20a after stimulation with IL-6 (P=0.033). Data are shown as means±SD derived from 4 independent experiments.

expression in HPAECs. Consequently, as shown in Figure 3C, the expression of STAT3 could be reduced by 50%. Stimulation experiments after established siRNA-mediated reduction of STAT3 are summarized in Figure 3D. Interestingly, the stimulatory effect of IL-6 on the expression of C13orf25 was almost completely abolished by the functional knockdown of STAT3 as compared with stimulated scrambled control cells (P=0.0027). Consistent with the Western blot (Figure 3B), the difference observed between siRNA and scrambled transfected cells when stimulated with VEGF was statistically significant but considerably weaker than the difference on stimulation with IL-6 (P=0.0428). To evaluate whether stimulation of HPAECs with IL-6 and VEGF might also affect the expression of mature miRNA derived from miR-17/92, expression levels of mature miR-20a were assessed and a significant upregulation was detected (1.44±0.31 fold; P=0.033; Figure 3E) as compared with control cells 24 hours after stimulation with IL-6. Expression levels of miR-20a measured after stimulation with VEGF, however, did not reach a statistically significant increase (1.44±0.61 fold; P=0.187). These data highlight the role of IL-6 as an inducer of mature miRNAs derived from the cluster miR-17/92.

Identification of a Highly Conserved STAT3-Binding Site in the Promoter of C13orf25

Because the previous experiments revealed an essential role of STAT3 for the induction of C13orf25, as a next step, a screening for STAT3-binding sites in the promoter of C13orf25 was assessed (TFsearch, Computational Biology Research Center, Advanced Industrial Science and Technology [AIST], Japan; http://www.cbrc.jp/research/db/TFSEARCH.html) and revealed a binding site app. Upstream (100 bp) of the respective start codon. Alignment of this region with several mammalian species is shown in Figure 4A and underscores the evolutionary conservation of this binding site. To confirm this potential binding site, promoter activity studies were performed. We thus inserted the promoter of C13orf25 upstream to a luciferase reporter construct (pGL3 basic promoter wild-type [WT]). In addition, the sequence of the predicted binding site was altered by introduction of three point mutations and used as control (pGL3 basic promoter ΔSTAT3). Transfection was performed in an IL-6–responsive tumor cell line (HepG2). Stimulation of these cells with IL-6 yielded a higher relative luciferase activity of the promoter WT (1.62±0.41 fold) as compared with the mutated promoter construct (ΔSTAT3,

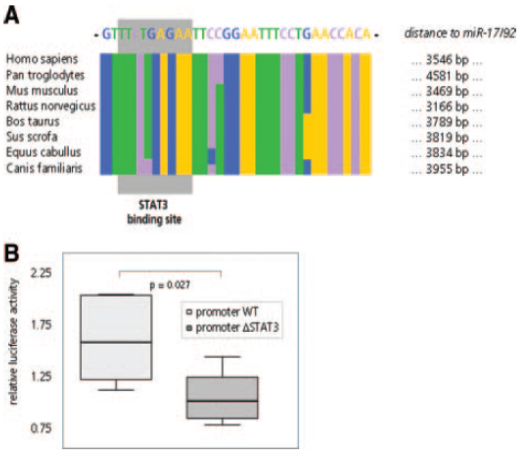

Figure 4. Identification of a highly conserved STAT3-binding site in the promoter of C13orf25. A, A 33-bp region in the promoter of C13orf25 contains a predicted STAT3-binding site. Alignment of this region with several mammalian species highlights the evolutionary conservation of this binding site (adapted from Loffler et al[20]). B, Reporter gene assay studies. Stimulation of HepG2 cells with IL-6 resulted in a higher relative luciferase activity of the promoter WT as compared with the mutated promoter construct (ΔSTAT3). Data are plotted as median including lower and upper whisker. Five independent experiments were performed.

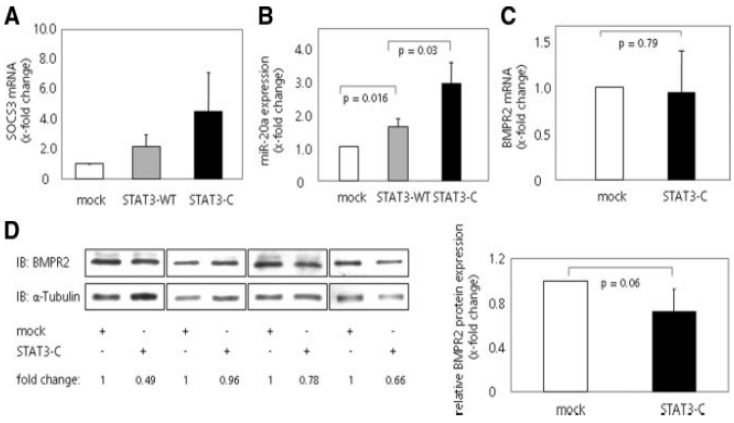

Figure 5. Transient transfection of constitutively active STAT3 downregulates BMPR2 in vitro. A, Quantification of SOCS3 mRNA in transfected HEK293 cells after 48 hours. Overexpression of both STAT3-WT and STAT3-C resulted in increased levels of SOCS3 as compared with mock transfected cells. B, Analysis of miR-20a expression performed in identical RNA extracts as in A. Levels of miR-20a were found upregulated in STAT3-WT and STAT3-C transfected cells when compared with mock transfected cells. C, Quantification of BMPR2 mRNA in STAT3-C–transfected HEK293 cells after 96 hours. The mRNA levels of BMPR2 was found unchanged as compared with mock. D, Western blot for BMPR2 in STAT3-C–transfected HEK293 cells after 96 hours showed a reduction of protein expression. Data are shown as means±SD derived from 4 independent experiments.

1.04±0.25 fold; P=0.027; Figure 4B), indicating the functional importance of this motif in the IL-6 mediated activation of C13orf25 transcription.

Transfection of Persistent Activated STAT3 Downregulates the Expression Levels of BMPR2 in an In Vitro System

To investigate the question whether the activation of STAT3 might affect the protein levels of BMPR2 through changes in the expression levels of miR-17/92, human STAT3, and a constitutively active form of human STAT3, STAT3-C, were cloned.[24] To prevent experimental cross-reaction by overexpression of STAT3, HEK293 cells were used that express endogenous STAT3 in low amounts only. As positive readout, the mRNA expression of suppressor of cytokine signaling (SOCS3), a well-known target gene of STAT3,[25] was quantified. Overexpression of the WT form of STAT3 resulted in increased levels of SOCS3 as compared with mock transfected cells (2.16±0.79 fold). This effect was even enhanced by the introduction of constitutively active STAT3-C (4.52±2.59 fold; Figure 5A), thus confirming the accurate construction of this molecule. The same samples were further analyzed for the expression of miR-20a as a representative of mature miRNAs derived from miR-17/92 (Figure 5B). Consequently, a similar expression pattern was seen in HEK293 cells for miR-20a as for SOCS3, showing significantly increased miR-20a levels in STAT3 WT transfected cells (1.6±0.24 fold; P=0.016) and STAT3-C transfected cells (2.89±0.63 fold; P=0.03). These data emphasize that the STAT3 pathway modulates the expression of mature miRNAs encoded by the miR-17/92 cluster.

The data presented so far showed that (1) the BMPR2 gene is regulated on a posttranscriptional level by miR-17-5p and miR-20a and (2) that the activation of STAT3 upregulates the preliminary transcript C13orf25 and the mature miR-20a. To link these findings, STAT3-C was overexpressed in HEK293 cells, and, subsequently, the BMPR2 expression was analyzed after 96 hours (Figure 5C). Analysis of the mRNA levels of BMPR2 revealed no significant changes (0.94±0.45-fold compared with mock) as shown in panel A. Conversely, the protein expression of BMPR2 was found to be reduced by ≈30% after constitutive activation of STAT3 (Figure 5D).

Discussion

In the present study, we found that (1) the protein expression of BMPR2 is modulated by the miR-17/92 cluster without affecting the BMPR2 mRNA levels; (2) this regulatory effect is driven by 2 distinct miRNAs, ie, miR-17-5 and miR-20a, through conserved seed matches within the 3′UTR of BMPR2; and (3) IL-6 regulates the expression of the miR-17/92 in HPAEC by signaling through STAT3. Moreover, we could show that (4) the promoter region of C13orf25 exhibits an evolutionary conserved STAT3-binding site and, finally, that (5) persistent activation of STAT3 leads to a strong upregulation of mature miR-20a, which, in turn, reduces the expression of BMPR2 protein. Taken together, our findings offer a novel mechanistic explanation for the downregulation of BMPR2, which has been repeatedly described as important feature in the pathogenesis of pulmonary hypertension.

The cell surface receptor BMPR2 is essential for the modulation of differentiation, proliferation and the fibrous matrix production of both endothelial and smooth muscle cells. [26,7] Changes in the expression of BMPR2 thus might promote vascular remodeling as observed in the arterial vessels of patients with pulmonary hypertension. This hypothesis is supported by the fact that the loss of BMPR2 resulting from germline mutations is a hallmark of genetic forms of pulmonary hypertension and, moreover, that reduced BMPR2 levels in the pulmonary arteries have been described in several animal models of pulmonary hypertension.[8,10] The regulation of BMPR2, however, is poorly understood to date, but results from previous studies suggest a posttranscriptional mechanism of regulation.[8,10] In this field, miRNAs have emerged as novel molecular players. To our knowledge, this study comprises the first data on the modulation of BMPR2 by miRNAs.

We approached the experiments by performing a computational screening that revealed multiple miRNAs as potential regulators of BMPR2. The highest prediction rates, however, were yielded for the miRNAs derived from the miR-17/92 cluster located in the third intron of the C13orf25 gene. It was

previously shown that this polycistronic region encodes for 6 mature miRNAs (ie, the miR-17/92 cluster: miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92).[18] To investigate the role of these miRNAs in the regulation of BMPR2, we used a mammalian pcDNA expression vector encoding for miR-17/92. Transient overexpression of this cluster led to a reduction of BMPR2 protein levels, whereas the mRNA levels remained unchanged. These results confirmed the hypothesis of a posttranscriptional role of miR-17/92 in the regulation of BMPR2. To prove whether these miRNAs directly interact with the 3′UTR of BMPR2, we further constructed a reporter gene system containing a luciferase gene and the predicted seed matches for miR-17/92. This assay showed repressed luciferase activity following overexpression of miR-17/92, thus verifying a direct binding of these miRNAs. Conversely, elevated levels of luciferase activity were found after blocking the endogenous miRNAs by using individually designed anti-miRs. In particular, we could show that the 3′UTR of BMPR2 is a direct target of 2 distinct miRNAs, ie, miR-17-5p and miR-20a, derived from the miR-17/92 cluster. Consistent with these data, TGFβR2, another receptor from the identical protein family, is also targeted by the miR-17/92 cluster.[13] TGFβR2 is a major mediator of tissue fibrosis and has been associated with the pathogenesis of pulmonary hypertension.[27] Moreover, it has been found that the TGFβR2 protein is almost absent in plexiform lesions that characterize the aberrant endothelial proliferation in idiopathic pulmonary arterial hypertension.[28] Whether the miRNAs derived from the cluster 17/92 regulate TGFβR2 and BMPR2 alike and, thus, might provide a final common pathway in the remodeling of pulmonary arterial vessels must be addressed by further studies.

The gene C13orf25 was first described as target for chromosomal amplification in malignant lymphoma.[29] This gene attracted primary attention after several studies suggested oncogenic activities probably attributable to targeting tumor suppressor genes, such as Bim and PTEN.[30] Moreover, Suarez et al showed that the miR-17/92 cluster is pivotally involved in the angiogenic sprouting of human endothelial cells.[31] Recent observations demonstrated that the expression pattern of miRNAs derived from miR-17/92 is regulated by a number of known transcription factors. O'Donnell et al, for example, described the modulation of miR-17/92 by c-Myc,[14] and the data on this regulatory network have recently been extended by Woods et al showing a direct interaction of E2F3 with the promoter region of C13orf25.[32]

In our experiments, we provide evidence that the promoter of C13orf25 also bears a functional binding site for STAT3, which controls the transcription of several genes involved in the inflammatory response.[33] By applying promoter studies, we confirmed a STAT3-responsive region located ≈100 bp upstream of the start codon of C13orf25. The functional importance of these findings is highlighted by the fact that this region was found to be phylogenetically conserved among mammalian species. In all species investigated, the distance of the STAT3-binding site to the respective sequence encoding for miR-17/92 revealed to span between 3200 and 4500 bp. Interestingly, a similar distance was described recently for the other known STAT3-regulated miRNA (ie, miR-21),[20] indicating a common evolutionary assembly of miRNA genes regulated by this transcription factor.

In normal cells, the expression and phosphorylation of STAT3 is finely balanced by negative feedback loops including the activation of SOCS proteins.[25] These feedback mechanisms, however, might be bypassed through persistent upstream signaling or through knock down of inhibitory proteins, leading to constitutively activated STAT3. Such phenomena have been found in several human tumors.[34] Of interest, a constitutive activation of STAT3 has also been described in human arterial endothelial cells derived from patients with pulmonary hypertension.[35] In general, the imitation of aberrant signaling by overexpression of persistently activated STAT3 displays an interesting experimental approach to identify novel miRNAs regulated directly or indirectly through inflammatory responses. The results obtained by such strategies might help to understand the reasons for altered miRNA expression profiles in these conditions. Along this line, we constructed such an expression vector for STAT3,[24] and subsequent transfection of this vector promptly resulted in increased levels of mature miR-20a as compared with mock or STAT3 WT transfected cells. Because this experimental setup reduced the expression of BMPR2 protein but not the respective mRNA, we suggest that this effect is driven, at least in part, by the upregulation of miRNAs derived from the cluster miR-17/92.

STAT3 was first described in the downstream signaling of IL-6 modulating acute phase gene expression.[36] Intriguingly, patients with pulmonary hypertension were found to have higher serum levels of IL-6 as compared with healthy controls,[3] and the ectopic administration of IL-6 has been observed to induce a mild elevation of the pulmonary arterial pressure in mice.[37,38] Moreover, the important role of IL-6 in the pathogenesis of pulmonary hypertension has been underpinned by a recent study in transgenic mice overexpressing IL-6. When compared with their WT counterparts, these animals developed increased ventricular systolic pressures, right ventricular hypertrophy, and pulmonary vasculopathic changes indicative for pulmonary hypertension.[5] In the present study, we thus investigated the influence of IL-6 on the expression of C13orf25 in HPAECs and found that IL-6 induced the gene expression of miR-17/92 in a STAT3-dependent manner.

Our data offer a direct link between the action of IL-6 and the expression of the miR-17/92 cluster. Because STAT3 has been shown to activate also the transcription of the c-myc gene,[39] our findings highlight the role of IL-6 in the regulatory pathway that controls the expression of miR-17/92 and thus complements this network by a novel piece (Figure 6).

Finally, our data allow us to conclude that increased IL-6 signaling leads to the downregulation of BMPR2, based on a phylogenetically conserved STAT3–miR-17/92 pathway. It could be speculated that STAT3 plays an important role in the development of pulmonary hypertension, in particular because it has been shown that a persistently activated STAT3 promotes cell survival of HPAECs derived from patients with pulmonary arterial hypertension. Because we could further show that STAT3 regulates the BMPR2 expression through transcriptional activation of miR-17/92, one might postulate

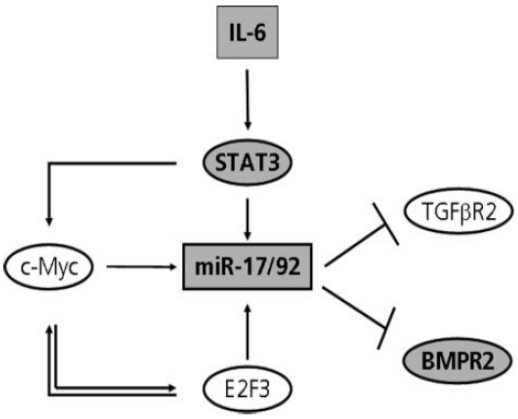

Figure 6. Mechanism of regulation of BMPR2. The transcription of miR-17/92 is regulated by c-Myc and E2F3.[14,32] In the present study, we demonstrate that the promoter region of miR-17/92 also bears a functional binding site for STAT3. The latent transcription factor STAT3 is the major mediator of IL-6 signaling. With respect to the fact that STAT3 induces the expression of c-Myc,[39] IL-6 emerges as a new modulator of miR-17/92. It is known that miRNAs derived from this cluster target the surface receptor TGFβR2.[13] Here, we provide evidence that the related BMPR2 is modulated by miR-17/92 alike.

this cluster as a highly specific target for the causative treatment of pulmonary hypertension. Because inhibitors of miRNAs are not presently available in a clinical setting, the inhibition of STAT3 activation by anticytokine therapies directed against IL-6 might provide a feasible alternative to restore functional levels of BMPR2.

Taken together, we provide here, to our knowledge, for the first time a mechanistic explanation for the loss of BMPR2 in pulmonary hypertension, shedding novel light on the pathogenesis of this disease and related conditions.

Sources of Funding

This work was funded by the Zurich Lung League Foundation and the Theodor and Ida Herzog-Egli-Foundation. M.B. is supported by the University Research Priority Program Integrative Human Physiology at the University of Zurich (ZIHP).

Disclosures

None.

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Supplementary information

Expanded materials and methods

Cell culture

Human embryonic kidney (HEK)293 cells and human hepatocellular carcinoma (HepG2) cells were grown in Dulbecco's minimum essential medium (Gibco-Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 50U/ml penicillin/streptomycin, 0.2% Fungizone, and 10mM HEPES (all reagents provided by Gibco-Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2. Human pulmonary artery endothelial cells (HPAEC) were purchased from Cascade Biologics (Invitrogen, Basel, Switzerland) and were cultured in Medium 200 (Invitrogen) according to the manufacturer's instructions. After an incubation period of 24h in starvation medium (0.5% FCS), recombinant human IL-6, VEGF or PDGF were added at a final concentration of 20ng/ml each. All growth factors and stimulation agents were purchased from R&D Systems (Abingdon, United Kingdom).

Plasmid construction

For overexpression of the miR-17/92 cluster, genomic DNA encoding miR-17/92 was amplified and cloned into pcDNA3.1 + (Invitrogen) between the *Hind*III and *Eco*RI restriction sites as previously described.[1] The following primers were used: sense 5'- AAA CTT AAG CTT CTA AAT GGA CCT CAT ATC TTT GAG - 3'; anti-sense 5'- CTG CAG AAT TCG AAA ACA AGA CAA GAT GTA TTT ACA C - 3'. The full-length sequence of human STAT3 was amplified out of cDNA derived from HepG2 cells and cloned into pcDNA3.1 + (Invitrogen) between the *Hind*III and *Xho*I restriction sites. The following primers were used: sense 5'- AAA CTT AAG CTT GCC GCC ATG GCC CAA TGG AAT CAG - 3'; anti-sense 5'- CTA GAC TCG AGT CAC ATG GGG GAG GTA GC - 3'. For construction of a constitutively active form of human STAT3, called STAT3-C,[2] the amino acid residues A661 and N663 were mutated to cysteine, respectively. The following primers were used: sense 5' - CAT GGA TTG TAC CTG CAT CCT GGT GTC TCC ACT G - 3'; anti-sense 5' - CCA GGA TGC AGG TAC AAT CCA TGA TCT TAT AGC - 3'. The correct sequence of each insert was confirmed by sequencing.

For transfection, HEK293 cells were seeded on 6- or 12-well plates at a density of 5 x 10^5 cells per ml in an antibiotic-free medium. The next day, cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Real time RT-PCR analysis

Total RNA was extracted using the RNeasy kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. Quantification of specific RNA transcripts was performed by SYBR Green real-time PCRs, using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). SYBR Green real-time PCR was performed for human bone morphogenetic protein receptor type II (BMPR2, sense 5' - AGC CCA ACA GTC AAT CCA ATG - 3'; anti-sense 5' - GGT TGC GTT CAT TCT GCA TAG - 3'), human suppressor of cytokine signaling 3 (SOCS3, sense 5' - CTG TAC CTG GGT GGA TGG AG - 3'; anti-sense 5' - TGA AAG ATG TCC CGT CTC CT - 3'), human signal transducer and activator of transcription (STAT3, sense 5' - TTC ACT TGG GTG GAG AAG GAC A - 3'; anti-sense 5' - CGG ACT GGA TCT GGG TCT TAC C - 3') and human preliminary transcript of the miR-17/92 cluster (C13orf25, sense 5' - TTG CTA AGT GGA AGC CAG AAG - 3'; anti-sense 5' - CAT CCA CGT GGC AAA ACA T - 3'). To confirm specific amplification by the SYBR Green PCR, a dissociation curve analysis was performed for each primer pair, and both non-RT negative controls and water controls were used for these analyzes. The amounts of loaded RNA were normalized by using a predeveloped 18S ribosomal RNA control kit (Applied Biosystems). Differential gene expression was calculated with the threshold cycle (C_t), and relative quantification was calculated with the comparative C_t method.

Quantification of mature microRNA-20a

Total RNA was extracted using the mirVana miRNA Isolation Kit (Applied Biosystems). Mature microRNA-20a was detected by stem-loop reverse transcription followed by SYBR Green real-time PCR,[3] using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Obtained signals were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, sense 5' - GGG AAG CTT GTC ATC AAT GGA - 3'; anti-sense 5' - TCT CGC TCC TGG AAG ATG GT - 3').

Western blot analysis

For protein extraction confluent cells were lysed using RIPA buffer (150mM NaCl, 50mM Tris-HCl, 250µM EDTA, 5mM NaF, 1% Triton X-100, 1% Deoxycholic acid). Whole-cell lysates (40 µg) were separated by 10% SDS-PAGE and the proteins were transferred to nitrocellulose or polyvinylidene fluoride membrane, respectively. The following antibodies were used for Western blot: anti - human BMPR2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti - human STAT3 (R&D Systems), anti - human Phospho-STAT3 (Tyr705, Cell Signaling Technology Inc., Beverly, MA, USA) and anti - α-Tubulin (Sigma, St. Louis, MO, USA). Evaluation of the expression of specific proteins was performed by the Alpha Imager Software system (Alpha Innotech, San Leandro, CA, USA) via pixel quantification of the electronic image.

Reporter gene assay

A 1554 bp fragment of the 3'untranslated region (UTR) of BMPR2 was amplified out of genomic DNA using the following primer: sense 5' - TAA TTC TAG AGC ATC ATT TAA ACA TGC AGA - 3'; anti-sense 5' - CGA CTC TAG ACA TCA GTT TGC AAA TTA ATA G - 3'. The PCR product was *Xba*I digested and cloned into the *Xba*I restriction site of the pGL3 control vector (Promega AG, Dübendorf, Switzerland). The correct sequence and orientation of the insert was confirmed by sequencing. As negative control, the anti-sense construct was used according to Kuhn et al.[4] For transfection, HEK293 cells were plated in 12-well plates at a density of 5 x 10^5 cells per ml in an antibiotic-free medium. The next day, cells were transfected with the pGL3 control 3'UTR of BMPR2 "sense" or "anti-sense" construct (150ng/well, respectively) using Lipofectamine 2000. Moreover, a vector encoding for the miR-17/92 cluster (pcDNA miR-17/92, 770ng/well) and a vector for normalisation (pRL-SV40, Promega; 80ng/well) were added. For inhibition of endogenous miRNAs, a similar protocol was applied with the use of anti-miRs (anti-miR-17-5p, anti-miR-19a, anti-miR-20a, anti-miR-92, all from Applied Biosystem; 100nM/well) instead of the miR-17/92 encoding vector. After 24h, the cells were lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The values obtained were normalized to the activity of Renilla luciferase (pRL-SV40). A 1351 bp fragment of the promoter of C13orf25 was amplified from human genomic DNA as previously described[5] using the following primer: sense 5' - GGG CTC GAG ATC TTT TCA GAT TTG GCC TTT TAT TTT - 3'; anti-sense 5' - AAT GCC AAG CTT AGG AGA GCT TCG CGG AGG AG - 3'.

The PCR product was digested with *Bgl*II and *Hind*III and cloned into the pGL3 basic vector (Promega). A mutation was introduced into the STAT3 binding site using the following primers: sense 5' - TAT GTC CTT GAG AAT TCC GGA ATT TCC TG - 3'; anti-sense 5' - TCT CAA GGA CAT AAT TGT TAA AAG TGA GG - 3' (alterations underlined). The correct sequence of each insert was confirmed by sequencing. For transfection, HepG2 cells were plated in 12-well plates at a density of 5 x 10^5 cells per ml in an antibiotic-free medium. The next day, cells were co-transfected either with the pGL3 basic promoter of C13orf25 wildtype (pGL3 basic promoter WT) or with the pGL3 basic promoter of C13orf25 ΔSTAT3 (pGL3 basic promoter ΔSTAT3, each 140ng/well) and pRL-SV40 (60ng/well) using FuGENE6 (Roche Diagnostics AG, Rotkreuz, Switzerland). After 6h, the cells were serum starved and stimulated with IL-6 (20ng/ml) for a time period of 4h. Luciferase activity was measured as mentioned above.

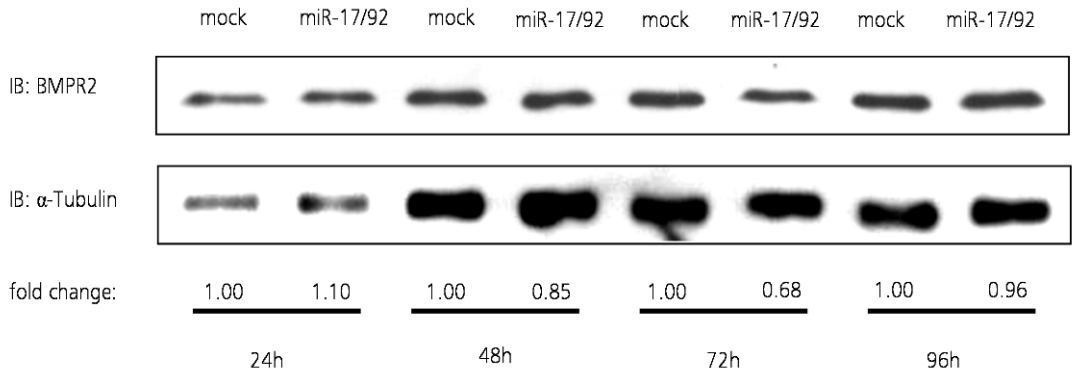
Nuclear transfection

To knock down the endogenous expression of the human STAT3 gene validated siRNA for STAT3 was purchased from Qiagen (Hs_STAT3_7 HP Validated siRNA). Nuclear transfection of HPAEC was achieved by using the nucleofection kit HMVEC-L from Amaxa (Amaxa GmbH, Cologne, Germany), according to the manufacturer's protocol. Briefly, cells were resuspended in nucleofection solution at a density of 5x10^5 cells per 100µl. For each transfection, 100µl of cell suspension were mixed with siRNA or scrambled negative control to obtain a final concentration of 100nM RNA. The suspension was pipetted into a cuvette and pulsed in a Nucleofector device using program S-05. Subsequently, cells were diluted with prewarmed medium and transferred into a 6-well plate. Following an incubation period of 24h, cells were serum starved and stimulated with the respective stimulation agent.

Statistics

For statistical analysis, GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used. To compare samples, the paired or unpaired t-test was applied and a p-value < 0.05 was considered to be statistically significant. All data are shown as mean ± SD.

Supplementary figure



Supplementary Figure S1. Over expression of miR-17/92 in HEK293 cells results in time-dependent downregulation of BMPR2.

HEK293 cells were transfected with the pcDNA miR-17/92 vector and protein was collected after 24h, 48h, 72h and 96h. Maximum effect of transient over expression of the miR-17/92 cluster was observed by Western blot analysis after 72h resulting in a decrease of app. 30% of BMPR2 protein.

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3. AntagomiR directed against miR-20a restores functional BMPR2 signaling and prevents vascular remodeling in hypoxia-induced pulmonary hypertension

Matthias Brock, Victor J. Samillan, Michelle Trenkmann, Colin Schwarzwald, Silvia Ulrich, Renate E. Gay, Max Gassmann, Louise Ostergaard, Steffen Gay, Rudolf Speich, Lars C. Huber. Submitted to the *European Heart Journal*

AntagomiR directed against miR-20a restores functional BMPR2 signaling and prevents vascular remodeling in hypoxia-induced pulmonary hypertension.

Matthias Brock[1,2], Victor J. Samillan[3], Michelle Trenkmann[1], Colin Schwarzwald[4], Silvia Ulrich[2], Renate E. Gay[1], Max Gassmann[3], Louise Ostergaard[3], Steffen Gay[1], Rudolf Speich[2], Lars C. Huber[1,2]

1) Center of Experimental Rheumatology and Zurich Center of Integrative Human Physiology (ZIHP) University Zurich, Switzerland

2) Pulmonary Hypertension Working Group, University Hospital Zurich, Switzerland

3) Institute of Veterinary Physiology, Vetsuisse Faculty and Zurich Center of Integrative Human Physiology (ZIHP), University Zurich, Switzerland

4) Equine Department, Vetsuisse Faculty, University Zurich, Switzerland

Abstract

Aims. Dysregulation of the bone morphogenetic protein receptor type 2 (BMPR2) is a hallmark feature that has been described in several forms of pulmonary hypertension. We recently identified the microRNA miR-20a within a highly conserved pathway as regulator of the expression of BMPR2. To address the pathophysiological relevance of this pathway *in vivo*, we employed antagomiR-20a and investigated whether specific inhibition of miR-20a could restore functional levels of BMPR2 and, in turn, might prevent pulmonary arterial vascular remodeling.

Methods and results. For specific inhibition of miR-20a, cholesterol modified RNA oligonucleotides (antagomiR-20a) were synthesized. The experiments in mice were performed by using the hypoxia-induced mouse model for pulmonary hypertension and animal tissues were analyzed for right ventricular hypertrophy and pulmonary arterial vascular remodeling. Treatment with antagomiR-20a enhanced the expression levels of BMPR2 in lung tissues; moreover, antagomiR-20a significantly reduced wall thickness and luminal occlusion of small pulmonary arteries and reduced right ventricular hypertrophy. To assess BMPR2 signaling and proliferation, we performed *in vitro* experiments with human pulmonary arterial smooth muscle cells (HPASMC). Transfection of HPASMC with antagomiR-20a resulted in activation of downstream targets of BMPR2 showing increased phosphorylation of Smad5 and activation of Id-1 and Id-2. Proliferation of HPASMC was found to be reduced upon transfection with antagomiR-20a.

Conclusion. This is the first report showing that miR-20a can be specifically targeted in an *in vivo* model for pulmonary hypertension. Our data emphasize that treatment with antagomiR-20a restores functional levels of BMPR2 in pulmonary arteries and prevents the development of vascular remodeling.

Introduction

Vascular remodeling of small pulmonary arteries is, together with vasoconstriction and microthrombosis, one of the pathogenetic hallmarks of pulmonary arterial hypertension (PAH). However, it is largely unknown what causes vascular remodeling or how it can be treated. Genetic mutations of the bone morphogenetic protein receptor type II (BMPR2), a member of the transforming growth factor (TGF-) β family expressed on the surface of endothelial and vascular smooth muscle cells of the pulmonary arterial circulation, have been described in familial and idiopathic PAH [1, 2]. Moreover, reduced expression of BMPR2 without concomitant mutations has also been found in other forms of pulmonary hypertension including established animal models of the disease [3, 4, 5]. It has been debated controversially whether the altered expression of BMPR2 due to mutations or downregulation might have a functional readout and, thus, an impact on the pathogenesis of PAH. Two recent studies however have underscored the potential role of BMPR2-mediated signaling for vascular remodeling [6, 7]. Long and coworkers observed that dysfunctional BMPR2/TGF-β signal transduction results in altered expression of transcription factors including Smads and Id (inhibitor of DNA binding) leading to migration of vascular smooth muscle cells [6]. Similar findings have been reported by another study showing that heterogeneous mutations of BMPR2 are associated with disrupted Smad signaling resulting
in a pro-proliferative and anti-apoptotic phenotype of pulmonary arterial smooth muscle cells [7].

MicroRNAs (miRNAs) comprise a class of small, non-coding RNA molecules that regulate gene expression on the post-transcriptional level [8]; miRNAs preferentially bind to conserved regions in the 3' untranslated region (UTR) of their target genes and either suppress the translation or the mRNA stability of these genes [9]. There is a growing body of evidence that the abnormal expression of miRNAs modulates human diseases by regulating key molecules of apoptosis, proliferation or cell signaling [10]. Conversely, efficient and non-toxic inhibition of miRNAs *in vivo* is of particular interest since such methods would offer a therapeutic approach to suppress altered miRNA expression in human disease. Krutzfeldt and co-workers

recently described the inhibition of miRNAs by the application of small anti-sense RNA molecules conjugated with cholesterol called antagomiRs in mice [11].

We have recently proposed that miRNAs might regulate the expression of BMPR2 and identified a phylogenetically conserved pathway involving the action of the signal transducer and activator of transcription (STAT)3 and the microRNA cluster miR-17/92, mainly of miR-20a [12]. Others have emphasised this hypothesis by showing that miRNAs derived from miR-17/92 are over expressed in experimental pulmonary hypertension *in vivo* [13] making these miRNAs interesting targets for further investigations. With respect to pathogenesis and therapy, this model offers the potential to restore functional expression levels of BMPR2 by antagonizing distinct miRNAs.

In the present study, we addressed feasibility and functional readout of specific antagonization of miR20-a by using antagomiR in an animal model of hypoxia-induced pulmonary hypertension *in vivo* and *in vitro*.

Material and methods

Materials and methods are provided as supplementary information.

Results

Application of antagomiR-20a suppresses miR-20a expression in target tissues

We recently identified a novel signaling pathway that regulates the expression of BMPR2 through the action of miR-20a [12]. Here we addressed the implications of this pathway in an animal model of hypoxia-induced pulmonary hypertension. The *in vivo* experiments were performed as described in the Methods (Fig. 1a). As shown in Figure 1b, repeated intraperitoneal injections of antagomiR-20a significantly downregulated the expression levels of miR-20a in lung tissues when assessed by qPCR at day 21. AntagomiR-20a treated animals showed an expression level of miR-20a of ΔCt 3.68 ± 0.58 as compared to ΔCt 2.31 ± 0.23 in normoxic control animals thus resulting in a reduction of 59.3% ($p < 0.001$). On the other hand, no significant changes were observed between normoxic control mice and antagomiR_mismatch (antagomiR_MM, ΔCt 2.46 ± 0.2) or PBS (ΔCt 2.18 ± 0.26) treated animals. Similar results were observed for other tissues analyzed, in particular heart and liver (data not shown). These data indicate that intraperitoneally injected antagomiRs are not subjected to a significant hepatic first-pass effect and, thus, are a feasible approach to inhibit

the expression of distinct miRNAs in target tissues including the lungs. The sequences of antagomiR-20a and antagomiR_MM are provided in Figure 1c.

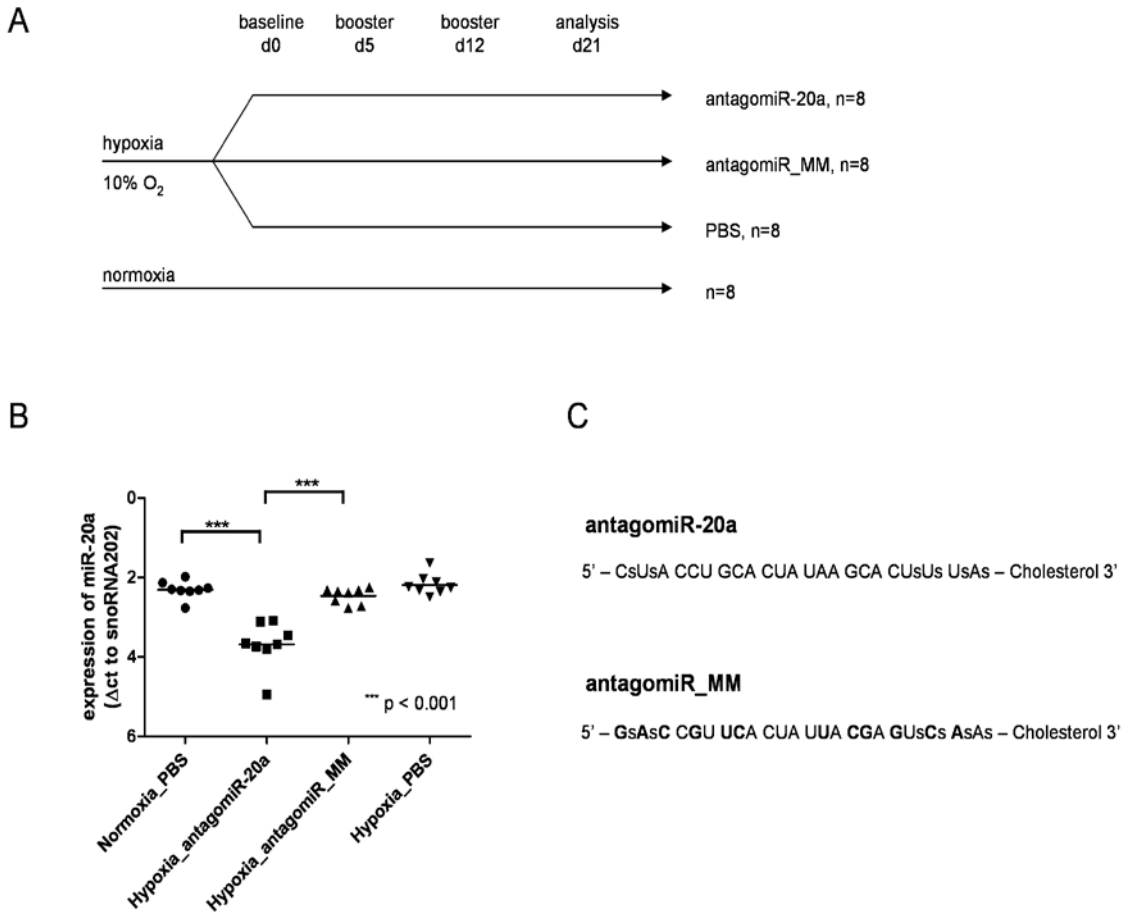


Figure 1. AntagomiR-20a suppresses miR-20a expression *in vivo.*

A. Study design of the *in vivo* experiments. 4 groups of 8 animals each were used; 3 groups were employed in conditions at 10% oxygen for up to 21 days and were treated with antagomiR-20a (25 mg/kg), antagomiR_MM or PBS. Injections were performed at day 0, 5 and 12. One group was used as normoxic control. B. miR-20a expression as normalized to snoRNA202 is significantly downregulated upon treatment with antagomiR-20a in lung tissue; statistical analysis by unpaired t-test. C. Sequence of antagomiR-20a and of the mismatch control, antagomiR_MM.

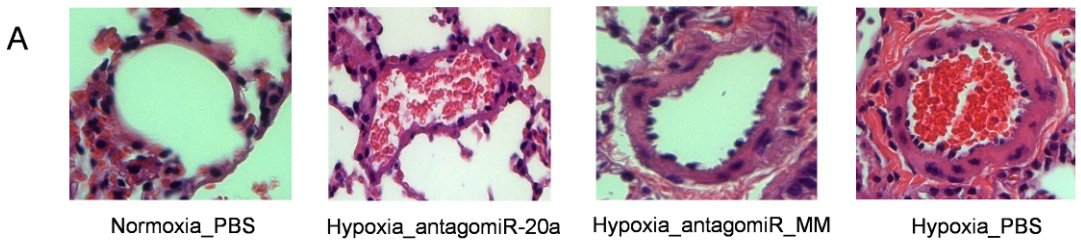
AntagomiR-20a prevents the development of hypoxia-induced morphological changes within the cardiopulmonary circulation

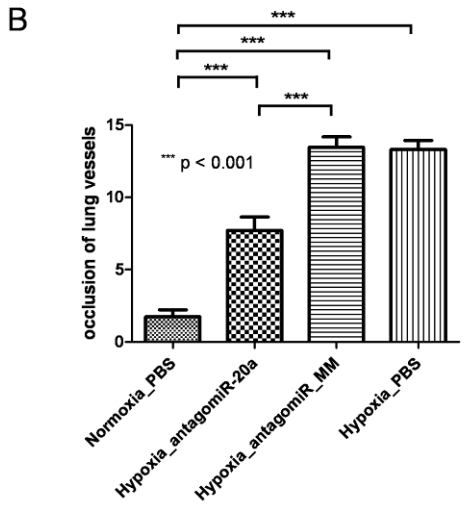
Morphometric analyzes were performed to assess the functional and hemodynamic consequences of reduced expression levels of miR-20a achieved by the application of antagomiR-20a. Representative images of small pulmonary arteries are shown in Figure 2A; treatment with antagomiR-20a strongly inhibited the vascular remodeling observed in PBS

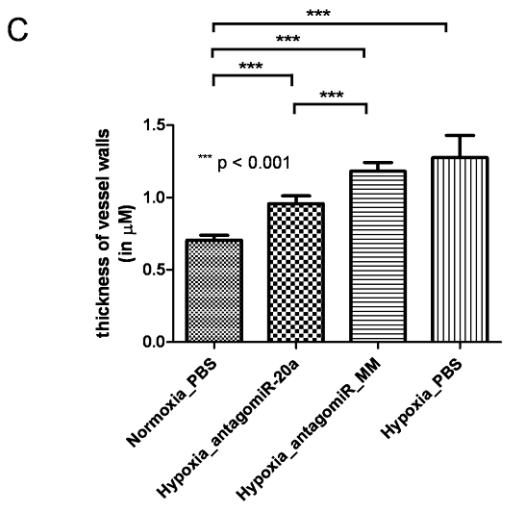
and antagomiR_MM treated hypoxic animals. Computer-based analysis further quantified the occlusion of these vessels to be reduced from 13.47 ± 0.73 and 13.33 ± 0.61 (antagomiR_MM and PBS treated animals, respectively) to 7.72 ± 0.93 in antagomiR-20a treated mice ($p < 0.001$) or to 1.73 ± 0.48 in normoxic controls (Fig. 2B). Similar effects were observed for the thickness of the vessel wall showing a reduction from 1.18 ± 0.06 μM and 1.28 ± 0.15 μM in antagomiR_MM and PBS treated animals to 0.96 ± 0.05 μM in antagomiR-20a treated animals (0.71 ± 0.04 μM in normoxic controls; Fig. 2C).

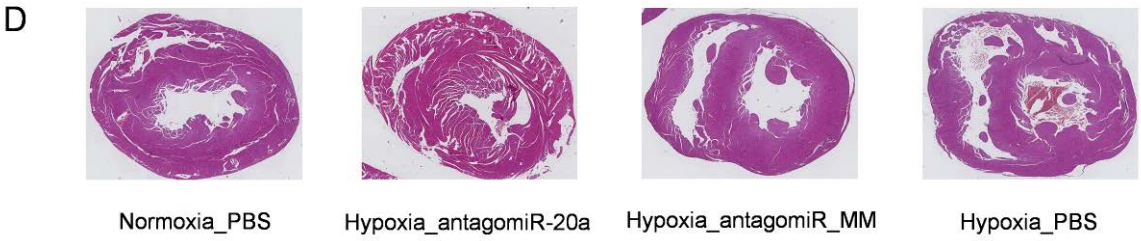
Right ventricular hypertrophy, assessed in Figure 2D, after 10% O_2 for 21 days was strongly reduced in animals receiving antagomiR-20a when compared to hypoxic mice treated with antagomiR_MM or PBS (representative images are shown in Fig. 2D). These results were confirmed by calculating heart weight relative to body weight (antagomiR_MM: 0.0061 ± 0.0006 vs. antagomiR-20a: 0.0056 ± 0.0007, $p = 0.091$; Fig. 2E) and by the relation of the right ventricular to the left ventricular volume (RV/LV + S; antagomiR_MM: 0.25 ± 0.01; antagomiR-20a: 0.21 ± 0.01, $p<0.001$; Fig. 2F), indicating that the right ventricular afterload was reduced in antagomiR-20a treated mice as compared to mock-treated hypoxic animals. Blood analysis performed at day 21 revealed that hypoxia resulted in erythrocytosis, which was most prominent in hypoxic PBS-treated animals (Fig 2G). Whereas significant differences were observed between normoxic controls (11.63 ± 0.91 g/dl) and all hypoxic groups (20.23 ± 2.04 g/dl for antagomiR-20a, 21.75 ± 1.28 g/dl for antagomiR_MM, and 23.27 ± 1.31 g/dl for PBS, respectively), differences between antagomiR-20a and antagomiR_MM treated animals did not reach statistical significance.

These data imply that the pathomorphological sequelae of hypoxia on the pulmonary vasculature, which lead to the development of pulmonary hypertension and, subsequently, to right ventricular hypertrophy, can be prevented by the application of antagomiR-20a.


A
Normoxia_PBS
Hypoxia_antagomiR-20a
Hypoxia_antagomiR_MM
Hypoxia_PBS


B
occlusion of lung vessels
*** p < 0.001
Normoxia_PBS
Hypoxia_antagomiR-20a
Hypoxia_antagomiR_MM
Hypoxia_PBS


C
thickness of vessel walls (in µM)
*** p < 0.001
Normoxia_PBS
Hypoxia_antagomiR-20a
Hypoxia_antagomiR_MM
Hypoxia_PBS


D
Normoxia_PBS
Hypoxia_antagomiR-20a
Hypoxia_antagomiR_MM
Hypoxia_PBS

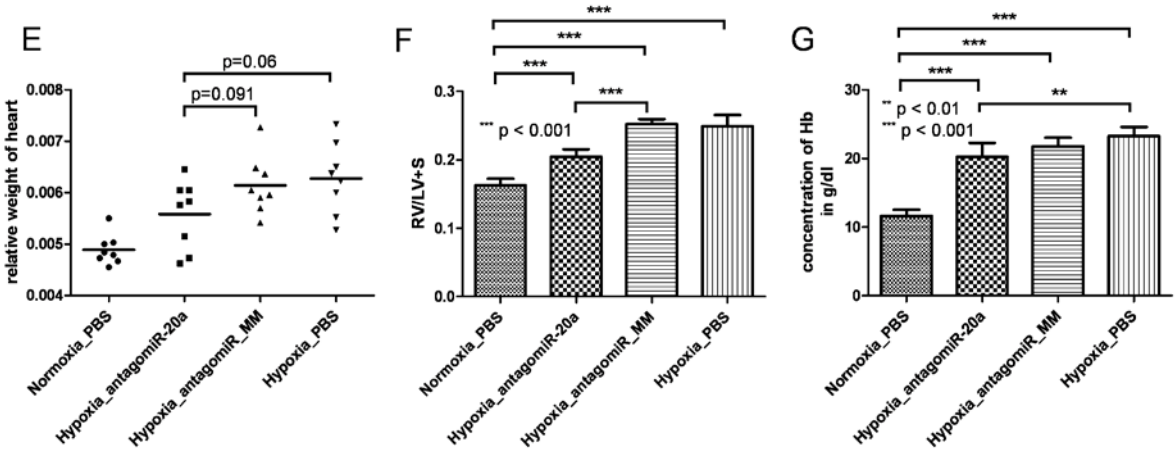

E
p=0.06
p=0.091
relative weight of heart
F
*** p < 0.001
RV/LV+S
G
** p < 0.01
*** p < 0.001
concentration of Hb in g/dl
Normoxia_PBS
Hypoxia_antagomiR-20a
Hypoxia_antagomiR_MM
Hypoxia_PBS

Figure 2. AntagomiR-20a prevents pulmonary arterial vascular remodeling.
A. Representative images of small pulmonary arteries showing reduced hypoxia-induced remodeling of intimal and medial vessel layer upon treatment with antagomiR-20a (second from left), quantified by analysis of lung vessel occlusion (B) and thickness of vessel wall (C). Similar findings were observed for heart morphometry as shown by representative images revealing reduced right ventricular hypertrophy by treatment with antagomiR-20a (D, second from left), heart weight relative to body weight (E) and heart hypertrophy (F). Hypoxia-induced erythrocytosis was not affected significantly by treatment with antagomiR-20a (G). Statistical analysis by unpaired t-test.

AntagomiR-20a restores functional expression levels of BMPR2 in mice

Since we hypothesized that the reduced expression levels of BMPR2 observed in pulmonary hypertension might be due to targeted inhibition by miR-20a [12], we next investigated whether the application of specific antagomiRs to mice increase BMPR2 expression levels, and whether restoration of this receptor might have functional implications. As shown in Figure 3a, the mRNA expression levels of BMPR2 in lung tissue were found to be significantly reduced in both hypoxic control groups (antagomiR_MM: ΔCt 3.36 ± 0.73; PBS: ΔCt 3.2 ± 0.41) when compared to normoxic mice (ΔCt 2.61 ± 0.19, $p = 0.021$ and $p = 0.004$, respectively). Interestingly, mRNA levels of BMPR2 under hypoxic conditions could be normalized to the levels observed in normoxic control animals by the application of antagomiR-20a (ΔCt 2.68 ± 0.16, Fig. 3a). These findings are further illustrated by immunohistochemical analysis using antibodies directed against BMPR2 showing that the application of antagomiR-20a increased the expression of BMPR2 on endothelial and smooth muscle cells of pulmonary arterioles as compared to antagomiR_MM injected mice (Fig. 3b).
Similarly, Id-2 and Smad5, two validated signaling elements downstream of the BMPR2 pathway [14] showed enhanced expression levels in lung tissues of animals treated with antagomiR-20a as compared to hypoxic controls (Smad5: antagomiR-20a ΔCt 3.89 ± 0.2 vs. hypoxia PBS ΔCt 4.16 ± 0.26, $p = 0.039$, Fig. 3c; Id-2: antagomiR-20a ΔCt 5.09 ± 0.46 vs. hypoxia PBS ΔCt 5.68 ± 0.52, $p = 0.031$; Fig 3d). These data suggest that treatment with antagomiR-20a increases expression levels of BMPR2 within the pulmonary vasculature and, most important, could restore functional BMPR2 signaling.

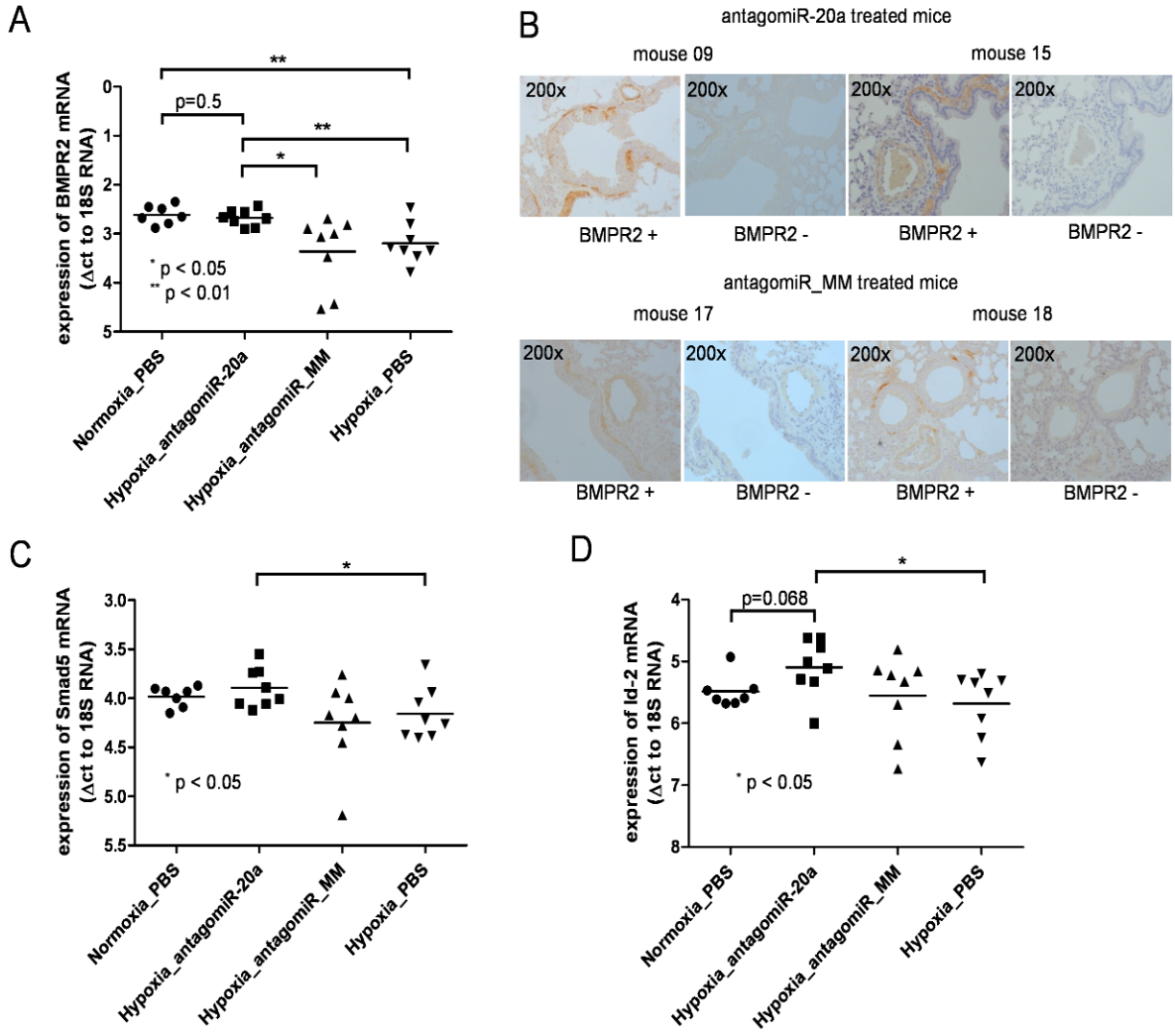


Figure 3. Restoration of functional expression levels of BMPR2 by antagomiR-20a in mice.
Treatment with antagomiR-20a increased mRNA expression of BMPR2 in lung tissue as shown by qPCR (A) and by immunohistochemistry (B, antagomiR-20a treated animals shown in upper panel, negative controls in lower panel. Magnification 20fold). Similarly, expression of Smad5 (C) and Id-2 (D) was significantly increased in lung tissue by treatment with antagomiR-20a. Statistical analysis by unpaired t-test.

AntagomiR-20a enhances BMP-2 mediated signaling in vitro

Upon binding of the ligand BMP-2 to BMPR2, Smad5 is activated by phosphorylation, which, in turn, activates the transcription factors Id-1 and -2. Since treatment with antagomiR-20a upregulated the expression levels of BMPR2 *in vivo*, we next investigated whether antagonization of miR-20a might also enhance the BMP-2 mediated signaling *in vitro*. We thus utilised human pulmonary arterial smooth muscle cells (HPASMC) that were stimulated

with BMP-2 for different time periods and found that treatment with antagomiR-20a enhanced BMPR2-induced signaling activity, i.e. phosphorylation of Smad5 as compared to antagomiR_MM controls (Fig. 4a). Phosphorylation of Smad5 (normalised to Smad5 protein levels) was increased in stimulated (e. g. after 15min of BMP-2 stimulation: antagomiR_MM, 1.53 ± 0.09 vs. antagomiR-20a, 1.83 ± 0.28) as well as in unstimulated conditions (antagomiR_MM, 1.11 ± 0.23 vs. antagomiR-20a, 1.61 ± 0.38, Fig. 4a).

Similar effects were seen for the expression levels of Id-1 and -2, showing an augmentation of the BMP-2 mediated induction of gene expression by transfection of antagomiR-20a. As shown in Fig. 4b, 1h of BMP-2 stimulation enhanced the mRNA levels of Id-1 in antagomiR-20a treated cells by 2.65 ± 1.55 fold (as compared to 1.52 ± 0.5 fold in antagomiR_MM transfected cells, p = 0.102). After 4h of BMP-2 stimulation Id-1 was significantly more induced in HPASMC treated with antagomiR-20a (4 ± 2.28) as compared to control cells (3 ± 2.14, p = 0.035). The mRNA levels of Id-2, on the other hand, were found to be significantly more elevated by BMP-2 stimulation in antagomiR-20a treated HPASMC after 1h only (antagomiR_MM: 1.14 ± 0.31 fold, antagomiR-20a: 1.65 ± 0.41 fold, p = 0.02, Fig. 4c).

Finally, promoter studies were performed to illustrate an enhancement of BMP-2 signaling by antagonizing miR-20a. The promoter of Id-1 containing multiple Smad transcription factor binding sites [15] was cloned into a luciferase-based reporter gene vector. As shown in Fig. 4e, 4h of BMP-2 stimulation induced the relative promoter activity of Id-1 by 1.38 ± 0.22 fold in antagomiR_MM transfected cells. Interestingly, the increase in promoter activity upon stimulation with BMP-2 was significantly more enhanced when cells were transfected with antagomiR-20a (1.69 ± 0.22 fold, p=0.016). Conversely, the over expression of miR-20a by transfection of small RNA oligonucleotides (mat-miR-20a) reduced the BMP-2 mediated induction of the promoter activity of Id-1 (mat-miR_MM: 1.81 ± 0.27 fold; mat-miR-20a: 1.47 ± 0.35 fold, p = 0.013).

Note that, Smad5 was predicted to be directly targeted by miR-20a (TargetScan, Whitehead Institute for Biomedical Research, www.targetscan.org, [16]). Accordingly, the expression of Smad5 after silencing of miR-20a in HPASMC was analyzed. As shown in Suppl. Fig. S1, transfection of antagomiR-20a significantly increased mRNA levels of Smad5 after 72h, but failed to enhance protein levels. Moreover, a reporter gene assay comprising the predicted seed match of miR-20a in the 3' untranslated region (UTR) of Smad5 could not show a direct miRNA - mRNA interaction (Suppl. Fig. S1).

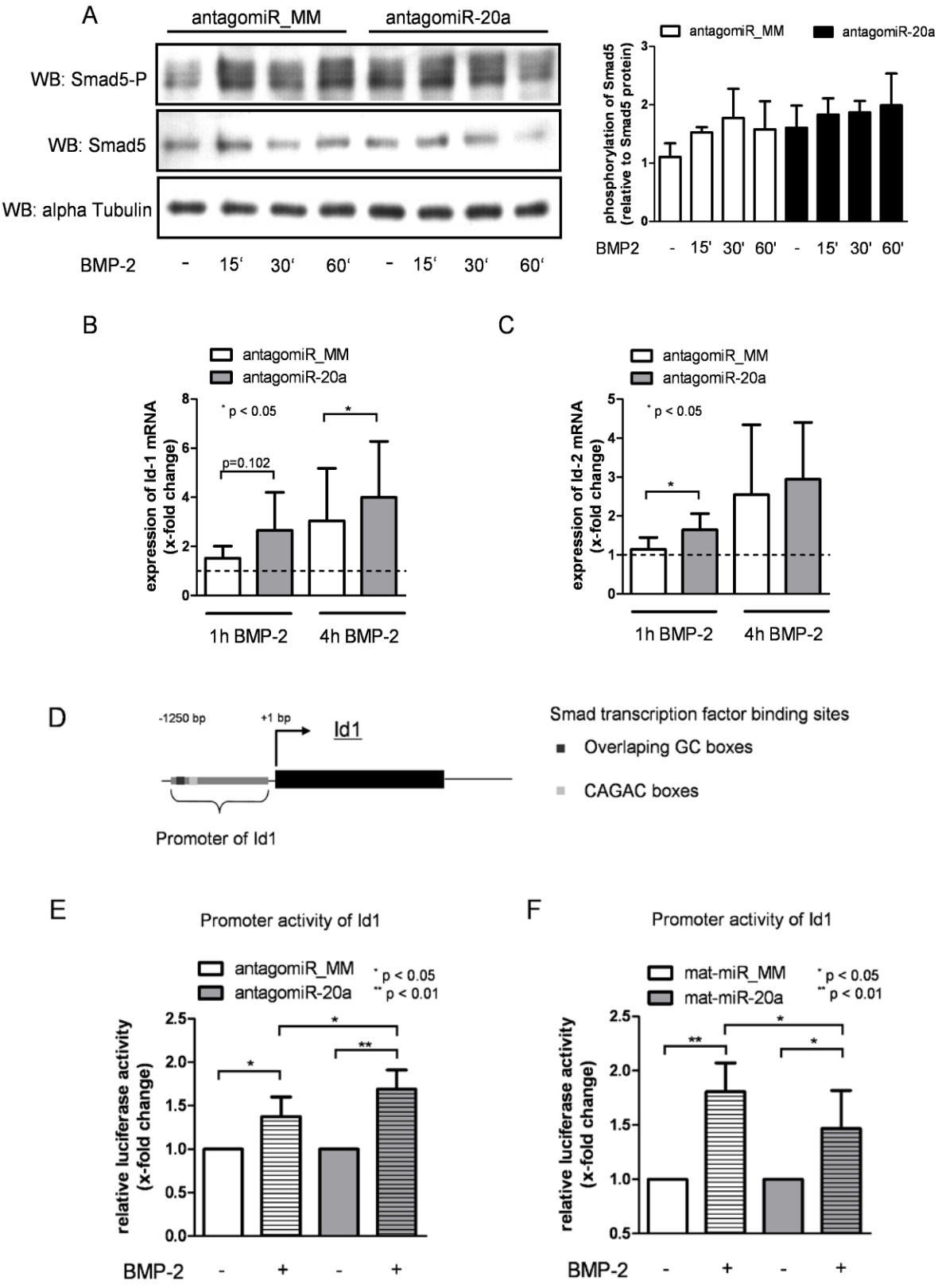


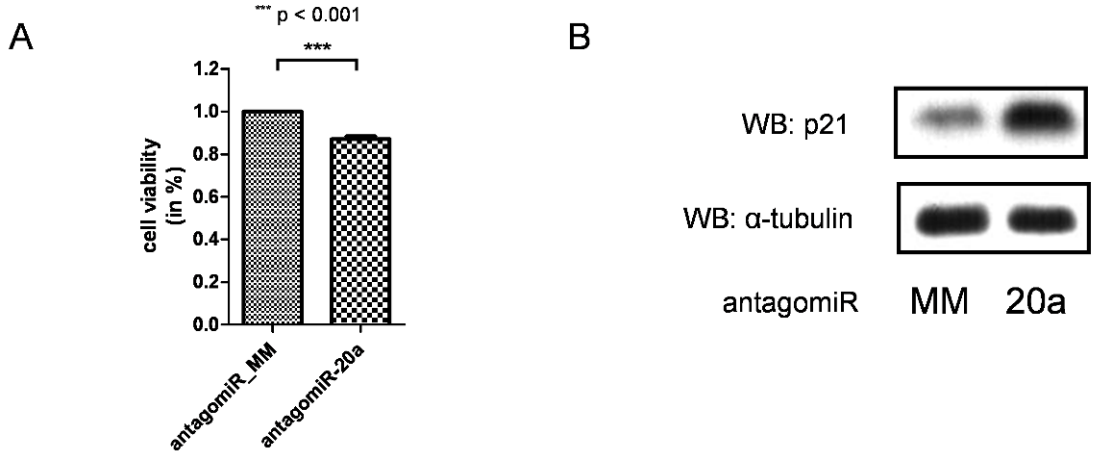
Figure 4. AntagomiR-20a enhances BMP-2 mediated signaling *in vitro.*

A. Smad5 phopshorylation in HPASMC treated with BMP-2 analyzed at different time points (15min, 30min, 60min) shows increased relative phosphorylation upon transfection with antagomiR-20a as

indicated by densitometric analysis (left panel). Expression of Id-1 (B) and Id-2 (C) is significantly increased after stimulation with BMP-2 when transfected with antagomiR-20a (assessed at 1 and 4h). Statistics by paired t-test, dashed line indicates unstimulated HPASMC. D. For promoter studies, the promoter of human Id-1 was cloned containing multiple Smad transcription factor binding sites (as described in Lopez-Rovira et al. [15]. E. Reporter gene assay in HepG2 cells. Co-transfection of pGL3basic-Id1 and antagomiR_MM or antagomiR-20a. Stimulation with BMP-2 for 4h increased response in antagomiR-20a treated cells. F. Conversely, the response to BMP-2 was reduced when miR-20a was over expressed. Statisitics by paired t-test.

Proliferation of vascular smooth muscle cells is reduced upon transfection with antagomiR-20a

Proliferation of pulmonary arterial smooth muscle cells is an essential part of the vascular remodeling in pulmonary hypertension; we thus addressed whether transfection with antagomiR-20a affects cell viability assessed by the MTT assay as surrogate for proliferation. As shown in Figure 5A, cell viability was reduced in antagomiR-20a transfected HPASMC by 12.9 ± 1.2 % as compared to antagomiR_MM transfected controls ($p < 0.001$). On the other hand, no changes were seen when apoptosis was analyzed (data not shown). AntagomiR-20a transfection of HPASMC resulted after 24h in upregulation of the cell cycle inhibitor p21 on protein level (Fig. 5B) as well as on mRNA level (1.53 ± 0.43 fold, $p = 0.051$ Fig. 5C). The expression of miR-20a was analyzed in the same samples showing downregulation of miR-20a in HPASMC by antagomiR-20a treatment (Fig. 5D).



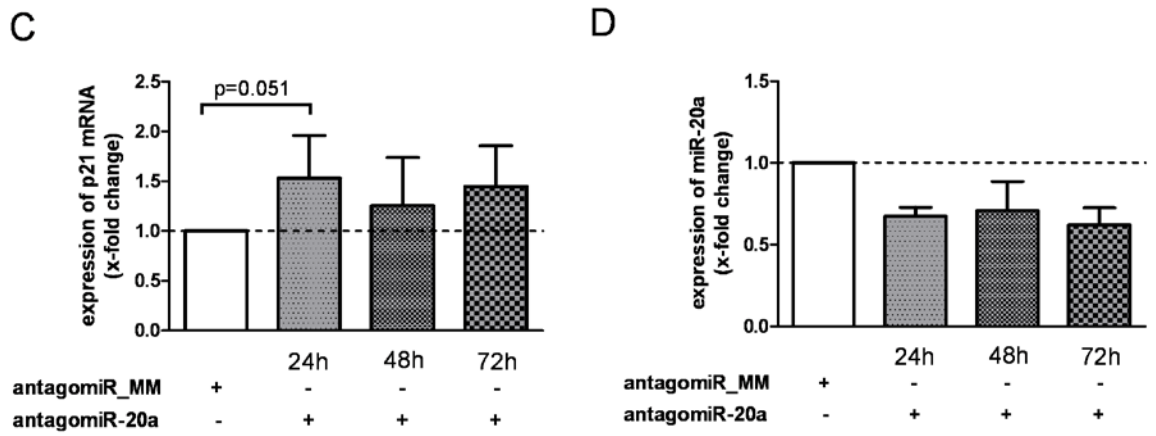


Figure 5. Cell proliferation is reduced upon transfection with antagomiR-20a.

A. MTT assay performed in HPASMC after transfection of antagomiR-20a and antagomiR_MM showed significant inhibition of cell growth. Statistics by paired t-test. B. p21 was found to be upregulated in antagomiR-20a transfected HPASMC as indicated by Western blot experiments (at 24h of antagomiR-20a transfection). C. mRNA levels of p21 were found to be increased in HPASMC when miR-20a expression was blocked as assessed by qPCR. Statistics by paired t-test. D. Proof of principle. miR-20a levels were found to be reduced upon transfection with antagomiR-20a.

Discussion

We have recently identified a microRNA-mediated signaling pathway that regulates the expression of BMPR2 on human pulmonary arterial endothelial cells [12]. While this pathway might explain, at least *in vitro*, the reduced expression of BMPR2 as observed in several forms of pulmonary hypertension, the functional relevance of this pathway was unclear so far. In the present study we thus employed an established animal model of pulmonary hypertension and found that i) intraperitoneal injection of antagomiR in general is a feasible approach to efficiently downregulate distinct miRNAs in target tissues; ii) antagonization of miR-20a reduces the hypoxia-induced remodeling of pulmonary arterioles and, subsequently, reduces right heart hypertrophy in hypoxic animals; and, iii), that treatment with antagomiR-20a restores functional expression levels of BMPR2 both *in vivo* and *in vitro*.

In the present experiments, antagonization of miR-20a significantly reduced the vascular remodeling in hypoxic animals as assessed by wall thickness and luminal occlusion of small pulmonary arteries. Thickness of the vessel wall was less prominent than in hypoxic control animals, probably due to a lesser grade of muscularization upon reduced proliferation of vascular smooth muscle cells in the medial layer. The vascular occlusion of these vessels that

is commonly observed in pulmonary hypertension and might result from vasoconstriction, proliferation of the intimal and medial layer and the development of plexiform lesions, has also been found to be reduced significantly in antagomiR-20a treated animals. Vascular occlusion and narrowing of the pulmonary arterial lumen increase pulmonary vascular resistance and right ventricular afterload; these alterations result in cardiac hypertrophy and, ultimately, right heart failure. In our experiments, antagomiR-20a treated animals revealed significantly less right ventricular hypertrophy than their mock-treated hypoxic controls, indicating improved pulmonary arterial hemodynamics in antagomiR-20a treated mice. However, since the expression of miR-20a has been antagonized not only in the lungs, it remains unclear whether the observed reduction of right ventricular hypertrophy is an indirect effect of reduced pulmonary arterial pressure or, alternatively, whether it might be due to concomitant inhibition of miR-20a in cardiac myocytes. In contrast to other miRNAs, including miR-133 [17] , miR-21 [18] or miR-27b [19] that also have been antagonized experimentally, miR-20a has not been described to be overexpressed in response to cardiac stress. With regard to other cell types, treatment with antagomiR-20a did not significantly alter the hypoxia-induced polycythemia of erythrocytes, indicating that, at least, no such pleiotropic effects by antagonizing miR-20a have occurred on hematopoietic cells.

We further showed that treatment with antagomiR-20a increased the expression levels of BMPR2 in lung tissue, thus confirming indirectly that miR-20a targets the mRNA of BMPR2 as it was suggested previously by our *in vitro* data on human pulmonary arterial endothelial cells. It has been discussed controversially, whether the observed dysregulation of BMPR2 in several forms of pulmonary hypertension might be of pathogenetic relevance. However, genetic studies and recent *in vitro* data suggest that BMPR2 and its downstream signaling play an important role in cell proliferation, vascular remodeling and, thus, in the development of pulmonary hypertension. For instance, Wong and co-workers showed that the activation of the BMPR2 signaling pathway in HPASMC leads to the inhibition of proliferation probably due to the induction of the cell cycle repressor p21 [20]. Our findings are along the line of these data and further provide evidence both *in vivo* and *in vitro* that a functional BMPR2 signaling prevents major vascular remodeling within small pulmonary arteries. In particular, we showed that the restoration of BMPR2 expression in lung tissue enhances the expression of Smad5 and, further downstream, leads to activation of the BMP-2 target Id-2. Similarly, *in vitro*, stimulation of HPASMC with BMP-2 and transfection with antagomiR-20a revealed increased BMP-2 signaling as indicated by enhanced promoter activity of Id-1 and

upregulated expression levels of Id-1 and -2. Since Smad5 could not be validated as a direct target of miR-20a (Suppl. Fig. S1) and, on the other hand, the ratio between phosphorylated and unphosphorylated Smad5 was found to be increased in stimulated and antagomiR-20a transfected cells, we suggest that the strengthened response of HPASMC to BMP-2 is directly due to an antagomiR-20a mediated increase of BMPR2 expression that enhances the phosphorylation activity upon binding of BMP-2. The transcription factors Id-1 and -2 have been associated with cell proliferation [21]; activated Id-1/-2 thus would reduce the proliferation rate of cells. Here, levels of Id-1 and -2 have been found to be increased in antagomiR-20a treated HPASMC, and, in subsequent experiments, the proliferation of HPASMC was reduced by transfection of antagomiR-20a. These data strongly indicate that the restoration of BMPR2 by treatment with antagomiR-20a is functional and, thus, reconstitutes the downstream signaling of BMPR2 in pulmonary arterial smooth muscle cells and in lung tissue. The reduced proliferation of smooth muscle cells was further shown to be associated with enhanced expression of the cell cycle repressor p21. We suggest that the observed increased expression of p21 by antagomiR-20a is caused by two ally acting mechanisms. On the one hand it was shown that miR-20a directly regulates the expression of p21 [22], and on the other hand Wong et al. demonstrated that BMP-2 signaling enhances protein levels of p21 [20]. Therefore, antagomiR-20a treatment increases p21 levels by the inhibition of miR-20a and by enhancing BMP-2 signaling, which might result in growth arrest of smooth muscle cells. In summary, functional restoration of BMPR2 appears to inhibit the development of hypoxia-induced vascular remodeling by enhanced intracellular signaling activity and reduced proliferation.

Finally, antagonization of miR-20a, one of the key microRNAs in pulmonary hypertension, was successfully achieved in hypoxic mice by repeated intraperitoneal injections. Since antagomiRs are employed to a growing extent for antagonization of distinct microRNAs for pathogenetic investigations and therapeutic purposes [11], these findings make antagomiRs to interesting tools for several reasons: downregulation of miR-20a was observed in target organs behind the liver, a finding that mutually excludes a significant hepatic first-pass effect; the effects have been longstanding, thus minimising the need for frequent applications; toxic effects have not been observed in terms of well-being of the animals or interferon-gamma expression in liver tissue (data not shown). Moreover, to our knowledge, this is the first report on intraperitoneal application of antagomiRs.

Taken together, we show here for the first time that the development of hypoxia-induced changes of small pulmonary arteries can be prevented by the application of antagomiR-20a, though it remains speculative whether this treatment would also reverse the once established disease. Our *in vivo* data, however, suggest that antagonization of miR-20a inhibits vascular remodeling of pulmonary arteries in patients with pulmonary hypertension and, thus, might provide a major impact on our understanding and treatment of this disease.

Acknowledgements

The authors thank Maria Comazzi for the excellent technical support with the immunohistochemistry.

This work was supported by the Zurich Lung League Foundation, the Schwyzer Stiftung, FP7 Masterswitch and IAR Epalinges.

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Supplementary information

Material and methods

Animal experiments

Male mice (BL6) were obtained by the Institute for Veterinary Physiology at the University of Zurich. A total of 40 animals was used, 8 of these were employed to adjust dosage and timing of the intraperitoneal (i.p.) applications of antagomiR-20a (pre-test). The study design of the *in vivo* experiments is provided in Figure 1a. Four groups were used (n= 8 each): 3 groups were employed in hypoxic conditions. The fraction of oxygen was decreased gradually from 21% to 10% over 60 minutes. Hypoxic conditions were provided by chambers connected to a gas mixer (Ruskinn Life Science). Animals were treated with antagomiR-20a (25 mg/kg), antagomiR negative control (antagomiR_MM) or PBS, respectively. Injections were performed at baseline (day 0) with boosters performed at day 5 and 12. One group of animals was used as normoxic controls. Mice were assessed daily for activity and well-being. Food and water was provided ad libitum. Blood gas analysis, morphometry and tissue preparation for later analysis were performed on day 21. The experiments were approved by Zurich Canton's Veterinary office. To assess morphometry, hearts and lungs were removed and stained with hematoxylin and eosin. Parameters including heart hypertrophy, cell wall thickness, and vessel occlusion were determined by using the MCID analysis program 7.0 (InterFocus Imaging, Cambridge, UK).

AntagomiR design

Cholesterol modified RNA oligonucleotides (antagomiRs) directed against human and murine miR-20a (MIMAT0000529) were designed as described ((1)) and synthesized by Microsynth (Microsynth, Balgach, Switzerland). As a negative control, twelve point mutations were introduced into the miR-20a mature sequence (antagomiR_MM) creating a RNA sequence which is not encoded in the murine or human genome. The sequences are the following: antagomiR-20a (5' – CUA CCU GCA CUA UAA GCA CUU UA – 3'), antagomiR_MM (5' – GAC CGU UCA CUA UUA CGA GUC AA – 3').

Cell culture

Human pulmonary arterial smooth muscle cells (HPASMC) were purchased from Invitrogen (Invitrogen AG, Basel, Switzerland) and were grown in supplemented Medium 231 (Invitrogen). Human hepatocellular carcinoma (HepG2) cells and human embryonic kidney (HEK)293 cells were grown in Dulbecco's modified essential medium (Invitrogen AG, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 50U/ml penicillin/streptomycin, 0.2% Fungizone, and 10mM HEPES (all reagents provided by Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2. BMP-2 (Invitrogen) was used for stimulation experiments of HPASMC and HepG2 cells (20ng/ml medium).

Quantitative real time-PCR (qPCR) analysis

Total RNA was extracted using the RNeasy kit (Qiagen AG, Hombrechtikon, Switzerland). RNA was reverse transcribed by using Random Hexamers and MultiScribe Reverse Transcriptase (both from Applied Biosystems, Rotkreuz, Switzerland). Quantification of specific RNA transcripts was performed by SYBR Green qPCR, using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Sequences of primers used in this study are shown in Suppl. Table S1. To confirm specific amplification, a dissociation curve analysis was performed. The amounts of loaded RNA were normalised to the expression of 18S RNA (Applied Biosystems). Differential gene expression was calculated with the threshold cycle (C_t) method (2).

Quantification of mature miRNAs

Total RNA was extracted using the miRNeasy miRNA isolation kit (Qiagen). Mature microRNA sequences were detected by specific stem-loop primers, reverse transcribed using MultiScribe Reverse Transcriptase and quantified by performing SYBR Green qPCR (3). Stem-loop primers and amplification primers were designed for miR-20a, snoRNA202, and RNU48 (Suppl. Table S1). Obtained signals were normalised to the expression of snoRNA202 (murine control) or RNU48 (human control) and specific amplification was confirmed by performing dissociation curve analysis.

Western blotting

For protein extraction, cells were lysed with sample loading buffer (62.5mM TrisHCl pH 6.8, 2% SDS, 10% glycerol, 5mM β-mercaptoethanol, bromphenolblue). Whole-cell lysates were

separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred to polyvinylidene fluoride membrane. Membranes were incubated with the following primary antibodies: anti - human Smad5, anti - Phospho-Smad5 (phospho S463 + S465; both from Abcam plc, Cambridge, UK), anti - human p21 (Cell Signaling Technology, Danvers, MA, USA), and anti - α-tubulin (Sigma, St. Louis, MO, USA or Abcam). Bands were detected with species-specific secondary antibodies coupled to horseradish peroxidase (Jackson ImmunoResearch Europe Ltd, Suffolk, UK). Evaluation of the expression of proteins was performed by the Alpha Imager software system (Alpha Innotech, San Leandro, CA, USA) via pixel quantification of the electronic image.

Plasmid construction

For promoter studies, a fragment (1247 bp) of the human Id-1 promoter containing Smad transcription factor binding sites (4) was amplified from human genomic DNA (Promega AG, Dübendorf, Switzerland) using PCR. The PCR product was digested with *Bgl*II and *Hind*III and cloned into the firefly luciferase-based pGL3basic vector (Promega). For normalisation of the luciferase activity, a modified vector encoding for *Renilla* luciferase (pRL-SV40, Promega) was used: the SV40 promoter of pRL-SV40 was removed by digestion with *Bgl*II and *Hind*III and replaced by the promoter of GAPDH (1063 bp). For confirmation of direct miRNA - mRNA interactions, the 3'untranslated region (UTR) of human Smad5 (1608 bp) was amplified from human genomic DNA using PCR. The obtained PCR product was digested with *Nhe*I and cloned into the pGL3control vector (Promega). Primer sequences used for cloning are shown in Suppl. Table S1. The correct sequence of each insert was confirmed by sequencing.

Reporter gene assay

For promoter activity studies, HepG2 cells were transfected with pGL3basic-Id1 (600ng) and pRL-GAPDH (300ng) using Lipofectamine 2000 (Invitrogen). In addition, RNA molecules for the inhibition (antagomiR-20a and, as negative control, antagomiR_MM) or for the overexpression of miR-20a (mat-miR-20a, sequence 5' – UAA AGU GCU UAU AGU GCA GGU AG – 3', and as negative control, mat-miR_MM, sequence 5' – GAC CGU UCA CUA UUA CGA GUC AA – 3') were co-transfected (all 100nM/well). After 48h, the cells were stimulated with BMP-2 (20ng/ml) for 4h, harvested, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The values obtained were

normalised to the activity of *Renilla* luciferase (pRL-GAPDH). For miRNA target validation, HEK293 cells were transfected with pGL3control-Smad5 (150ng) and pRL-SV40, 80ng) using Lipofectamine 2000. Moreover, RNA molecules for the inhibition of miR-20a (antagomiR-20a and, as negative control, antagomiR_MM) were added. After 24h, the cells were lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System. The values obtained were normalised to the activity of *Renilla* luciferase (pRL-SV40).

Transfection of antagomiRs in vitro

For specific knock down of miR-20a in HPASMC, antagomiR-20a (100nM) was transfected using Lipofectamine 2000. AntagomiR_MM (100nM) served as negative control. Following an incubation period of 72h, cells were stimulated with BMP-2 (20ng/ml) for different time points (as indicated), harvested, and gene expression analysis was performed.

Cell viability assay

To assess cell viability, HPASMC were seeded in 24-well plates and were transfected with antagomiR-20a or antagomiR_MM (100nM) using Lipofectamine 2000. After 72h, 100µl of Thiazolyl Blue Tetrazolium Bromide solution (MTT, 5mg/ml, Sigma) was added to each well and incubated for 7h. Purple MTT formazan crystals were dissolved in acidified isopropanol and spectrophotometrically measured at a test wavelength of 560 nm. The measured absorbance directly correlates with the viability and the number of cells.

Immunohistochemistry

To illustrate expression of murine BMPR2 *in vivo* , formalin-fixed paraffin embedded tissue sections were stained with goat - anti - BMPR2 (sc-5682, Santa Cruz Biotechnology, Santa Cruz, CA, USA), developed with 3,3'-diaminobenzidine and counter stained with hematoxylin and eosin. Normal goat IgG was used as negative control.

Statistics

For statistical analysis, GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used. To compare samples with parametric distribution, the paired or unpaired t-test was applied and a p-value < 0.05 was considered to be statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All data are shown as mean ± SD.

Supplementary figures and tables

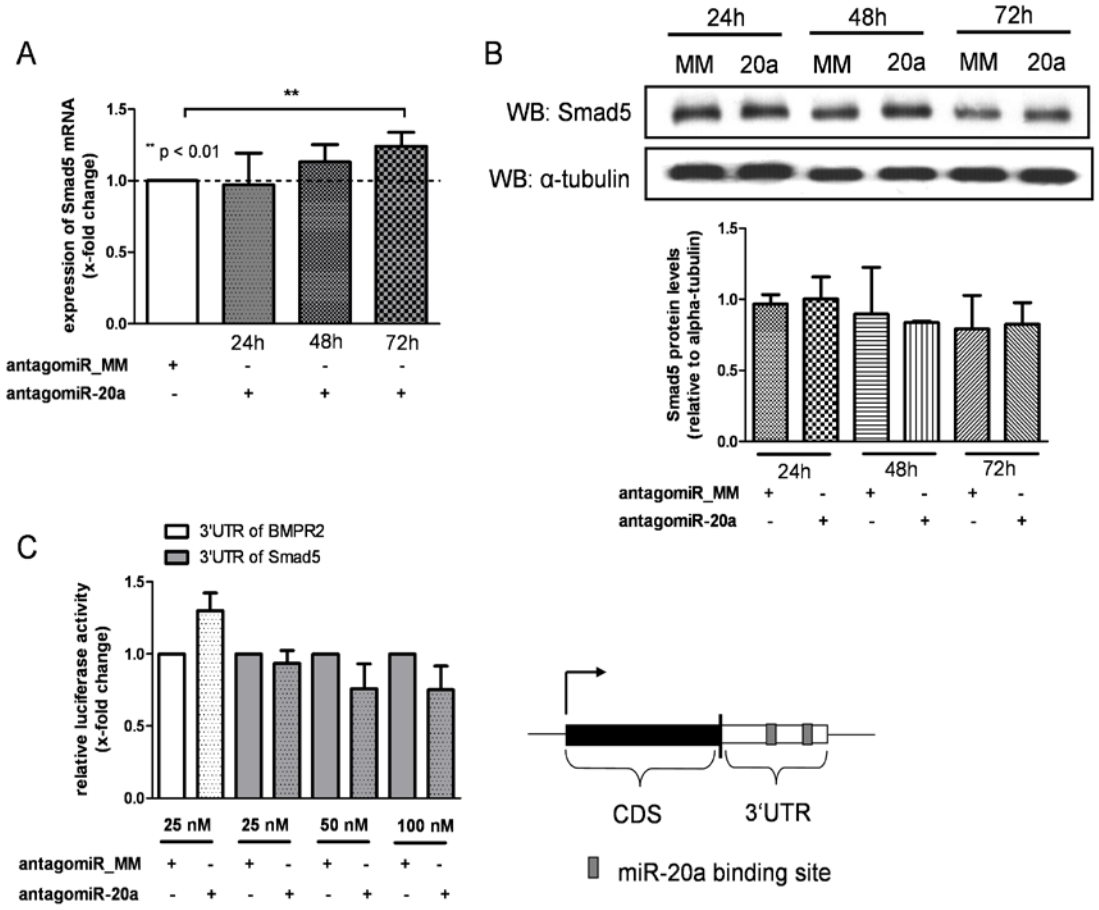

Figure S1. Human Smad5 is not a direct target of miR-20a.

The expression of miR-20a in HPASMC was blocked by transfection of antagomiR-20a. Treatment with antagomiR_MM served as negative control. (A) Inhibition of miR-20a after 72h resulted in significantly increased mRNA levels of Smad5 as assessed by qPCR (n = 5). (B) Western blot showed no upregulation of Smad5 on protein levels in cells transfected with antagomiR-20a (n = 4). One representative Western blot is shown. (C) HEK293 cells were co-transfected with a reporter gene vector containing the 3'UTR of Smad5 (illustration is shown) and antagomiR_MM or antagomiR-20a. Inhibition of miR-20a by antagomiR-20a did not upregulate relative luciferase activity indicating no direct miRNA – mRNA interaction. As a positive control a reporter gene assay containing the 3'UTR of BMPR2 (described in Brock et al., (5)) was used (n = 3). Statistical analysis by paired student's t-test (antagomiR_MM control transfection was set to 1).

Table S1. Primer sequences

gene expression analysis	
BMPR2 (mmu) fwd	5’ – CCA CGA GGA GAT CAT TAT AAA CC– 3’
BMPR2 (mmu) rev	5’ – ACA TTC CTG CTG TTT AAA TCT CG – 3’
Id-2 (mmu) fwd	5’ – CCT TGC AGG CAT CTG AAT TCC – 3’
Id-2 (mmu) rev	5’ – GTA CTT TGC TAT CAT TCG ACA TAA G – 3’
Smad5 (mmu, hsa) fwd	5’ – AGA GTC CAG TCT TAC CTC CAG – 3’
Smad5 (mmu, hsa) rev	5’ – AGG CTG TGT TGT GGA TTG AAT TC – 3’
Id-1 (hsa) fwd	5’ – AAG GTG AGC AAG GTG GAG ATT C – 3’
Id-1 (hsa) rev	5’ – CAA CTG AAG GTC CCT GAT GTA G – 3’
Id-2 (hsa) fwd	5’ – GAA TAA GCG GTG TTC ATG ATT TC – 3’
Id-2 (hsa) rev	5’ – GAT TCC GTG AAT TTG TTG TTG TTG – 3’
CDKN1A (p21, hsa) fwd	5’ – AGC ATG ACA GAT TTC TAC CAC TC – 3’
CDKN1A (p21, hsa) rev	5’ – GGC TTC CTC TTG GAG AAG ATC – 3’
miRNA expression analysis	
miR-20a RT (stem loop primer)	5’ – GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT ACC TG – 3’
miR-20a fwd	5’ – GCG GCG GTA AAG TGC TTA TAG TG – 3’
miR-20a rev	5’ – TGC AGG GTC CGA GGT AT – 3’
RNU48 RT (stem loop primer)	5’ – GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GTC AG – 3’
RNU48 fwd	5’ – CCA TGA GTG TGT CGC TGA TG – 3’
RNU48 rev	5’ – GTG CAG GGT CCG AGG T – 3’
plasmid construction	
Id-1 promoter (*BglII*) fwd	5’ – GGG CTC GAG ATC TTG CAG AGC TGG AAA GAG AAC – 3’
Id-1 promoter (*HindIII*)	5’ – AAT GCC AAG CTT AGT GCG GAG CCA CAG

rev	CTT – 3’
3'UTR of Smad5 (*NheI*)	5’ – TAA TGC TAG CCA ATT ATA TTG TTA GTG GAC
fwd	TTG – 3’
3'UTR of Smad5 (*NheI*)	5’ – CGA CGC TAG CGA GAG AAG AGT CTA TAT
rev	TCG – 3’

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4. MicroRNA-18a enhances the IL-6 mediated production of the acute-phase proteins fibrinogen and haptoglobin in human hepatocytes

Matthias Brock[1,2], Michelle Trenkmann[1], Renate E. Gay[1], Steffen Gay[1], Rudolf Speich[2], Lars C. Huber[1,2]. *J Biol Chem* **2011**, in Press. Published on September 27, 2011 as Manuscript M111.251793

Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2011/09/27/M111.251793.DC1.html

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 46, pp. 40142–40150, November 18, 2011

MicroRNA-18a Enhances the Interleukin-6-mediated Production of the Acute-phase Proteins Fibrinogen and Haptoglobin in Human Hepatocytes*[S]

Received for publication, April 19, 2011, and in revised form, September 6, 2011 Published, JBC Papers in Press, September 27, 2011, DOI 10.1074/jbc.M111.251793

Matthias Brock[‡§1], Michelle Trenkmann[‡], Renate E. Gay[‡], Steffen Gay[‡], Rudolf Speich[§], and Lars C. Huber[‡§]

From the [‡]Center of Experimental Rheumatology and Zurich Center for Integrative Human Physiology (ZIHP), University Zurich and the [§]Working Group for Pulmonary Hypertension, University Hospital Zurich, CH-8091 Zurich, Switzerland

The acute-phase response is an inflammatory process triggered mainly by the cytokine IL-6. Signaling of IL-6 is transduced by activation of STAT3 (signal transducer and activator of transcription 3), which rapidly induces the production of acute-phase proteins such as haptoglobin and fibrinogen. Another target of the IL-6/STAT3 signal transduction pathway is the microRNA cluster miR-17/92. Here, we investigated the interplay of miR-17/92 and STAT3 signaling and its impact on the acute-phase response in primary human hepatocytes and hepatoma (HepG2) cells. Employing a reporter gene system consisting of STAT3-sensitive promoter sequences, we show that the miR-17/92 cluster member miR-18a enhanced the transcriptional activity of STAT3. IL-6 stimulation experiments in miR-18a-overexpressing hepatocytes and HepG2 cells revealed an augmented acute-phase response indicated by increased expression and secretion of haptoglobin and fibrinogen. This effect was due, at least in part, to repression of PIAS3 (protein inhibitor of activated STAT, 3), a repressor of STAT3 activity, which we identified as a novel direct target of miR-18a. Finally, we demonstrate that the expression of miR-17/92 in primary hepatocytes and HepG2 cells is modulated by IL-6. Our data reveal, for the first time, a microRNA-mediated positive feedback loop of IL-6 signal transduction leading to an enhanced acute-phase response in human hepatocytes.

Severe inflammation is observed in several conditions, including overwhelming infection, systemic inflammatory response syndrome, sepsis, and septic shock, and results in the activation of a signaling cascade that is commonly referred to as the acute-phase response. Acute-phase proteins are involved in a great variety of physiological and biochemical processes, including growth inhibition of microbes (*e.g.* complement factors), blood coagulation (*e.g.* fibrinogen), and binding of proteins (*e.g.* haptoglobin-mediated binding of hemoglobin) (1). During inflammation, the production of plasma proteins by hepatocytes is altered either by increasing the levels of plasma proteins (positive acute-phase reaction) or by decreasing their levels (negative acute-phase reaction).

IL-6 is one of the most important cytokines that orchestrates the hepatic production of acute-phase proteins (2). For signal transduction, IL-6 binds to a membrane-associated receptor complex, which subsequently leads to the phosphorylation of Tyr-705 of STAT3 (signal transducer and activator of transcription 3) (3). Phosphorylated STAT3 is actively transported to the nucleus, where it activates the transcription of IL-6 target genes such as the acute-phase genes haptoglobin (Hp)[2] (4) and fibrinogen γ-chain (FGG) (5). Several negative feedback loops antagonize the activation of STAT3 to avoid persistent downstream signaling and subsequent dysregulation in the production of acute-phase proteins. A well characterized feedback pathway includes the blocking of the phosphorylation of STAT proteins by the action of the cytokine-inducible family of SOCS (suppressors of cytokine signaling) (6).

MicroRNAs (miRNAs) comprise a novel class of short non-protein-coding RNAs that regulate the expression of their target genes in a post-transcriptional manner either by mRNA degradation or by translational repression (7). The importance of miRNAs in the signal transduction of IL-6 has been highlighted by a recent study showing that miRNAs derived from the miRNA cluster miR-17/92 modulate STAT3 phosphorylation in multiple myeloma cells (8); moreover, Fort *et al.* (9) identified several miRNAs that are directly involved in the production of the acute-phase protein fibrinogen in human hepatoma (HuH-7) cells. The polycistronic miRNA cluster miR-17/92 codes for six mature miRNAs (miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92) and has primarily attracted attention for being frequently linked to cancer by exhibiting oncogenic activities mainly due to targeting tumor suppressor genes (10). The expression of miR-17/92 is regulated by a number of known transcription factors, including the oncogenic transcription factor c-Myc (11). In a previous work, we provided evidence that, upon stimulation with IL-6, the expression of miR-17/92 is directly regulated by STAT3, thus emphasizing a potential link between miR-17/92 and inflammatory processes (12). On the basis of these findings, we hypothesized that miR-17/92 might represent a key component

* This work was supported by Swiss National Foundation Grant SNF-32000-116842, the Schwyzer Foundation, the Zurich Lung League Foundation, and the Masterswitch FP7 and Institute for Arthritis Research Epalinges Programs.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Materials and Methods," Figs. S1–S3, Table S1, and additional references.

[1] To whom correspondence should be addressed: Center of Experimental Rheumatology and Working Group of Pulmonary Hypertension, University Hospital Zurich, Gloriastr. 23, CH-8091 Zurich, Switzerland. Tel.: 41-44-255-3729; Fax: 41-44-255-4170; E-mail: matthias.brock@usz.ch.

[2] The abbreviations used are: Hp, haptoglobin; FGG, fibrinogen γ-chain; miRNA, microRNA.

in IL-6 signal transduction contributing to the regulation of the acute-phase response in hepatocytes.

Because hepatocellular carcinoma (HepG2) cells have been extensively used to investigate the IL-6-induced expression of acute-phase proteins and thus provide an established model to study the acute-phase response (13), we used these cells in this study to address the role of miR-17/92 in the expression of acute-phase genes and to identify novel miR-17/92 targets in the IL-6 signaling cascade. In addition, the interaction between miR-17/92 and the acute-phase response was investigated in primary human hepatocytes to underscore the role of these miRNAs in a physiological setting. Our data reveal, for the first time, a miRNA-mediated positive feedback loop of IL-6 signal transduction leading to an enhanced acute-phase response in human hepatocytes.

EXPERIMENTAL PROCEDURES

Methods—These are described in detail under supplemental "Materials and Methods."

Cell Culture—HepG2 human hepatocellular carcinoma cells and HEK293 human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Primary human hepatocytes were purchased from Lonza (Verviers, Belgium) and cultured according to the manufacturer's instructions. For stimulation experiments, HepG2 cells and primary hepatocytes were serum-starved for 24 h and treated with IL-6 as indicated.

Quantitative Real-time PCR Analysis—Quantification of specific mRNA transcripts was performed by SYBR Green quantitative real-time PCR using the ABI Prism 7700 sequence detection system (Applied Biosystems, Rotkreuz, Switzerland) with normalization to the expression of GAPDH. Mature miRNAs were detected by specific stem-loop primers according to Chen *et al.* (14). Primer sequences are shown in supplemental Table S1.

Western Blotting—The following primary antibodies were used: anti-PIAS3 (protein inhibitor of activated STAT, 3) and anti-Hp (both from Abcam, Cambridge, United Kingdom), anti-STAT3 (R&D Systems), anti-phospho-STAT3 (Tyr-705; Cell Signaling Technology, Danvers, MA), and anti-α-tubulin (Sigma). Bands were detected with species-specific secondary antibodies coupled to horseradish peroxidase and quantified with AlphaImager software (Alpha Innotech, San Leandro, CA).

Plasmid Construction—An 878-bp fragment of the Hp promoter containing two STAT3-binding sites and a 578-bp fragment of the FGG promoter containing one STAT3-binding site were each cloned into the firefly luciferase-based pGL3-Basic vector (Promega, Dübendorf, Switzerland). The SV40 promoter of pRL-SV40 (Promega) was replaced with the promoter of GAPDH (1063 bp). The 3′-UTR of PIAS3 (924 bp) was cloned into the pGL3-Control vector (Promega). As a negative control, an antisense construct was used (15), as well as a construct with the specifically mutated seed match for miR-18a.

Reporter Gene Assay—For promoter activity studies, HepG2 cells were transfected with pGL3-Basic-Hp or pGL3-Basic-FGG and pRL-GAPDH for normalization. After 24 h, the cells were stimulated with IL-6 for 4 h. Luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega) and normalized to the activity of *Renilla* luciferase.

For miRNA target validation, HEK293 cells were transfected with pGL3-Control-PIAS3–3′-UTR, pRL-SV40 for normalization, and precursor molecules (pre-miRNAs). After 24 h, the cells were lysed, and luciferase activity was measured using the Dual-Luciferase reporter assay system.

Transfection of siRNAs and miRNAs—HepG2 cells were transfected with 200 nM siRNAs (Qiagen, Hombrechtikon, Switzerland) or 100 nM pre-miRNAs or anti-miRNA (Ambion/Applied Biosystems; pre-miR-18a, pre-miR-19a, pre-miR-20a, pre-miR-92a, or anti-miR-18a) using the cell line Nucleofector kit V (Amaxa, Cologne, Germany) or Lipofectamine 2000. Following an incubation period of 48 or 72 h, cells were stimulated with IL-6 (20 ng/ml).

ELISA—To determine levels of Hp and fibrinogen in cell culture supernatants, we used commercially available ELISA kits (GenWay Biotech Inc., San Diego, CA) according to the manufacturer's instructions.

Statistics—For statistical analysis, GraphPad Prism software was used. To compare samples, Student's paired or unpaired *t* test was applied, and a p value <0.05 was considered to be statistically significant. All data are shown as means ± S.D. or as single raw data (primary hepatocytes).

RESULTS

miR-17/92 Enhances the Acute-phase Response in HepG2 Cells—This study was dedicated to investigating the role of miR-17/92 in IL-6 signaling and acute-phase response. Recently, we demonstrated that STAT3 directly activates the transcription of miR-17/92 upon IL-6 stimulation in human pulmonary arterial endothelial cells (12). To address the question of whether IL-6 enhances the expression of miR-17/92 in HepG2 cells and primary hepatocytes similarly, IL-6 stimulation experiments were performed. As shown in Fig. 1*A*, HepG2 cells were stimulated for 1 h with different amounts of IL-6 ranging from 5 to 100 ng/ml, and expression of the primary transcript of miR-17/92 (*C13orf25*) was assessed as described by O'Donnell *et al.* (11). Treatment with the lowest dose of IL-6 led to a small but significant up-regulation of *C13orf25* (1.19 ± 0.13-fold, $p = 0.008$) (Fig. 1*A*); the strongest induction of *C13orf25* mRNA levels was found when stimulated with 50 ng/ml IL-6 (1.54 ± 0.41-fold, $p = 0.012$).

Dose-dependent induction of two acute-phase genes (*i.e.* Hp and FGG) used as positive controls is shown in Fig. 1 (*B* and *C*, respectively). These data reflect the potential of IL-6 to efficiently stimulate the production of acute-phase genes in HepG2 cells.

Next, we used primary hepatocytes to address the role of IL-6 and miR-17/92 in another experimental model of the acute-phase response. We thus measured the expression levels of three miRNAs derived from miR-17/92 (miR-18a, miR-19a, and miR-20a) 24 h after IL-6 treatment and found that the expression levels of these miRNAs were increased in both donors used in this study (Fig. 1*D*). Together with our previous findings (12), these data support a cell type-independent mechanism for the induction of miR-17/92 by IL-6.

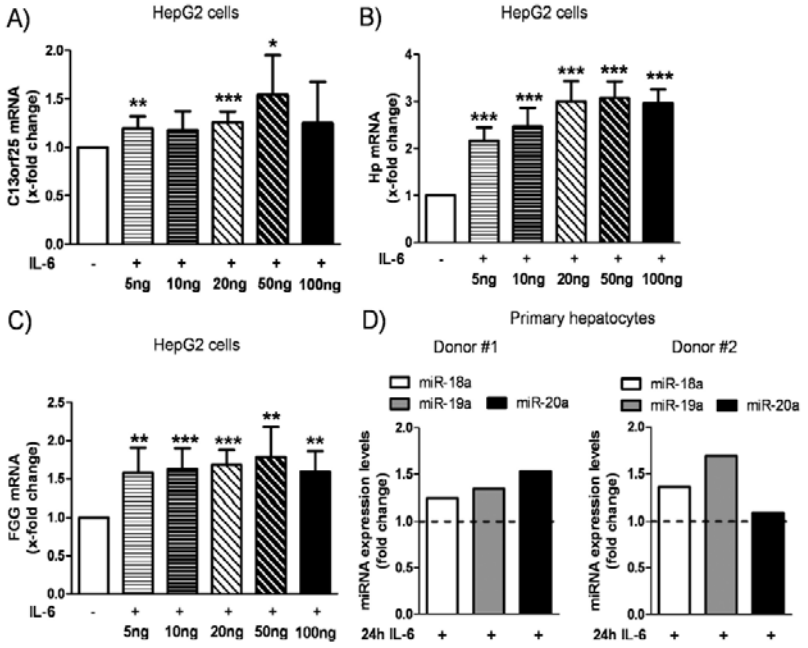

FIGURE 1. **Dose-dependent induction of *C13orf25* and acute-phase genes by IL-6.** HepG2 cells and primary hepatocytes were stimulated with IL-6 for 1 or 24 h, respectively. *A*, the mRNA levels of *C13orf25* were dose-dependently induced by IL-6, with 50 ng/ml IL-6 yielding the strongest increase in *C13orf25* ($n = 7$). *B* and *C*, IL-6 induced the mRNA expression of the acute-phase genes Hp and FGG ($n = 7$), respectively. *D*, IL-6 stimulation (20 ng/ml) for 24 h led to increased expression levels of miR-18a, miR-19a, and miR-20a in primary hepatocytes ($n = 2$). The *dashed lines* indicate unstimulated control cells. The respective unstimulated samples were set to 1. Statistical analysis was by Student's paired *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

In further study, we investigated the functional consequence of enhanced expression of miR-17/92 in HepG2 cells, in particular the significance for IL-6 signaling and acute-phase response. We performed gain-of-function experiments by transfecting HepG2 cells with precursor molecules of miR-18a, miR-19a, miR-20a, and miR-92a (representing the four functional miRNA families encoded by miR-17/92) and a mixture of all four. Successful overexpression of miRNAs was confirmed by quantitative real-time PCR (supplemental Fig. S1). The impact of each miRNA on the activity of STAT3 was measured employing a luciferase-based reporter gene system consisting of STAT3-sensitive promoter sequences of two selected acute-phase genes, *i.e.* Hp and FGG. In scrambled transfected cells, IL-6 increased the relative Hp promoter activity by 15.48 ± 4.51-fold compared with unstimulated scrambled transfected cells (Fig. 2*A*). Interestingly, the increase in promoter activity upon stimulation with IL-6 was significantly more enhanced when HepG2 cells were transfected with miR-18a (26.40 ± 4.47-fold, $p = 0.002$), miR-20a (21.32 ± 3.04-fold, $p = 0.032$), or a mixture of all four miRNAs (24.4 ± 5.46-fold, $p = 0.011$). Similar results were obtained when the promoter activity of FGG in HepG2 cells was analyzed, showing a significantly enhanced IL-6 response after transfection of miR-18a, miR-20a, miR-92a, and the miR-18a/miR-19a/miR-20a/miR-92a mixture compared with scrambled transfection (Fig. 2*B*).

To confirm the results of the reporter gene assay, the mRNA levels of both acute-phase genes in HepG2 cells were quantified. IL-6 stimulation for 4 h thus induced the expression of Hp by 4.43 ± 1-fold in scrambled transfected cells. Overexpression of miR-18a (6.31 ± 0.75-fold, $p = 0.001$), miR-20a (5.92 ± 1.17-fold, $p = 0.005$), miR-92a (5.9 ± 0.6-fold, $p = 0.005$), or the miRNA mixture (5.57 ± 1.09-fold, $p = 0.046$) significantly enhanced the induction of Hp transcripts (Fig. 2*C*). Quantification of FGG mRNA levels yielded a similar expression pattern (Fig. 2*D*). After 4 h of stimulation with IL-6, the expression of FGG was induced in miR-18a-transfected cells (3.89 ± 1.35-fold, $p = 0.082$) and miR-20a-transfected cells (3.64 ± 0.51-fold, $p = 0.03$) compared with scrambled transfection (2.86 ± 0.93-fold).

Overexpression of miR-18a Promotes the Expression of Acute-phase Proteins—Because miR-18a and miR-20a showed the most consistent and prominent effects in modulating the acute-phase response at the mRNA level, we assessed the secretion of fibrinogen and Hp into the supernatants of HepG2 cells stimulated with IL-6 for 8 or 24 h. Fibrinogen release from miR-18a-transfected cells collected 8 h after stimulation was significantly increased both in unstimulated cells (2170 ± 602 ng/ml, $p = 0.018$) and in IL-6-stimulated cells (2760 ± 702.6 ng/ml, $p < 0.001$) compared with scrambled transfection (unstimulated, 1669 ± 312.1 ng/ml; IL-6-stimulated, 2194 ± 556.3 ng/ml) (Fig. 3*A*). Enforced expression of miR-20a did not influence the release of fibrinogen either in IL-6 naive cells or under stimulated conditions. Similarly, after 24 h of IL-6 stimulation, miR-18a-overexpressing cells produced significantly more fibrinogen (4899 ± 253.2 *versus* 4017 ± 173.5 ng/ml in the scrambled control, $p = 0.002$) (Fig. 3*A*), whereas transfection of miR-20a failed to enhance the release of fibrinogen.

The same supernatants were assessed for Hp protein release, and, again, we found that miR-18a overexpression increased the secretion of the acute-phase protein (Fig. 3*B*). In detail,

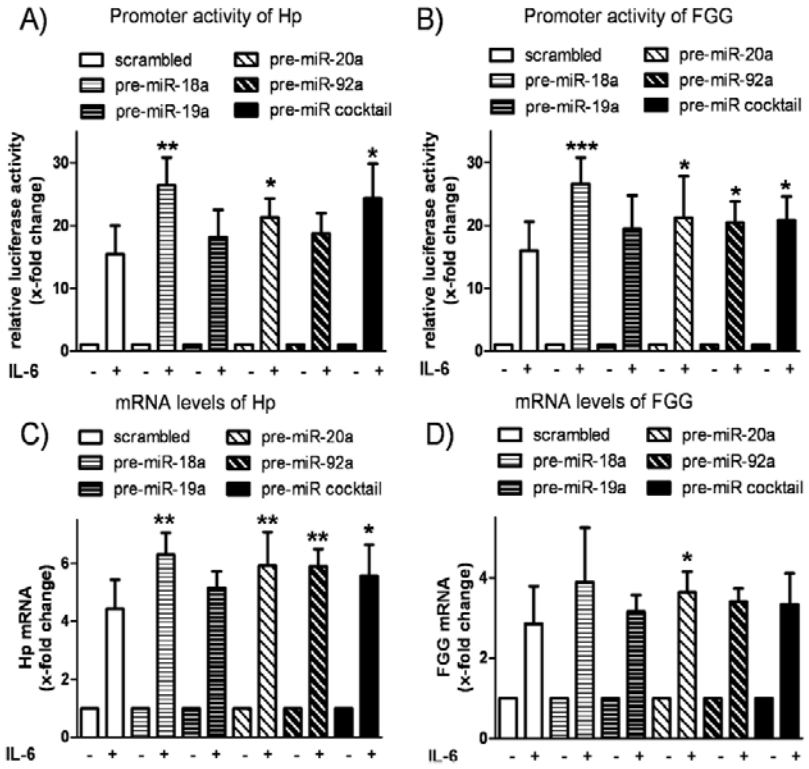

FIGURE 2. **Effect of miR-17/92 on acute-phase response in HepG2 cells.** HepG2 cells were transfected with pre-miRNA (pre-miR-18a, pre-miR-19a, pre-miR-20a, pre-miR-92a, and a mixture of all four) or a scrambled negative control. *A*, the promoter activity of Hp was assessed by cotransfection of a luciferase-based vector system comprising 878 bp of the Hp promoter sequence. 4 h of IL-6 stimulation (20 ng/ml) induced relative luciferase activity in all samples, whereas the IL-6 response was more pronounced in miR-18a-, miR-20a-, and miR-18a/miR-19a/miR-20a/miR-92a-transfected cells ($n = 5$). *B*, a luciferase-based reporter gene system consisting of the promoter sequence of FGG (578 bp) was cotransfected in HepG2 cells. The response to 4 h of IL-6 stimulation was enhanced by cotransfection of miR-18a, miR-20a, miR-92a, and the mixture of all four miRNAs compared with the scrambled control ($n = 7$). *C*, quantification of the mRNA levels of Hp after 4 h of IL-6 stimulation (20 ng/ml) showed a similar result as the respective reporter gene studies. The IL-6-inducible effect on Hp transcription was significantly enhanced by transfection of miR-18a, miR-20a, miR-92a, and the mixture of all four miRNAs ($n = 6$). *D*, identical samples show significant alteration of FGG mRNA expression by IL-6 in miR-20a-transfected cells ($n = 6$). The respective unstimulated samples were set to 1. Statistical analysis was by Student's unpaired *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

miR-18a significantly enhanced Hp release after both 8 h (166.6 ± 41.46 ng/ml, $p = 0.02$) and 24 h (420.8 ± 11.43 ng/ml, $p < 0.001$) of IL-6 stimulation compared with scrambled transfected and IL-6-stimulated cells (139.9 ± 32.6 and 368.7 ± 5.25 ng/ml, respectively). Hp release in miR-20a-overexpressing cells remained unaffected. To confirm these findings, identical experiments were performed in primary hepatocytes. As shown in Fig. 3*C*, the IL-6-induced release of fibrinogen after 24 h was more pronounced in hepatocytes transfected with precursor molecules of miR-18a compared with the scrambled control. Similarly, we found increased expression of Hp in miR-18a-overexpressing hepatocytes as indicated by Western blotting (Fig. 3*D*).

The ELISA and Western blot experiments thus confirmed the potential of miR-18a (but not of miR-20a) to promote the expression and release of acute-phase proteins. Conversely, the loss-of-function experiments by antagonizing the expression of miR-18a led to a decreased acute-phase response in HepG2 cells (supplemental Fig. S2).

miR-18a Directly Targets PIAS3—The previous experiments revealed an enhanced hepatic acute-phase response upon overexpression of miR-18a. Because miRNAs have been implicated in the repression of gene expression, we speculated that miR-18a might target a negative regulator of IL-6 signaling. Utilizing a computational screening (TargetScan, Whitehead Institute for Biomedical Research) (16), the 3′-UTR of PIAS3 was identified to contain a potential binding site for miR-18a. PIAS3 is endogenously expressed in HepG2 cells and specifically inhibits the DNA-binding activity of STAT3 (17). To investigate whether miR-18a influences PIAS3 expression, we measured the mRNA levels of PIAS3 in miR-18a-transfected HepG2 cells and primary hepatocytes. As shown in Fig. 4*A*, miR-18a transfection significantly reduced the expression of PIAS3 mRNA in HepG2 cells (by 36 ± 7%, $p < 0.001$) and in hepatocytes from both donors. Consistent with these data, Western blot experiments showed reduced protein levels of PIAS3 in HepG2 cells transfected with pre-miR-18a (PIAS3/α-tubulin ratio, 0.52 ± 0.04 (scrambled transfection) and 0.35 ± 0.12 (pre-miR-18a transfection), $p = 0.044$) (Fig. 4*B*). To prove the specificity of the observed reduction of PIAS3, we additionally quantified PIAS3 protein expression in miR-20a-transfected cells. miR-20a, which was not predicted to target PIAS3, did not change the protein expression of PIAS3 (PIAS3/α-tubulin ratio, 0.53 ± 0.09, $p = 0.773$) (Fig. 4*B*), implying an efficient and specific down-regulation of PIAS3 by miR-18a.

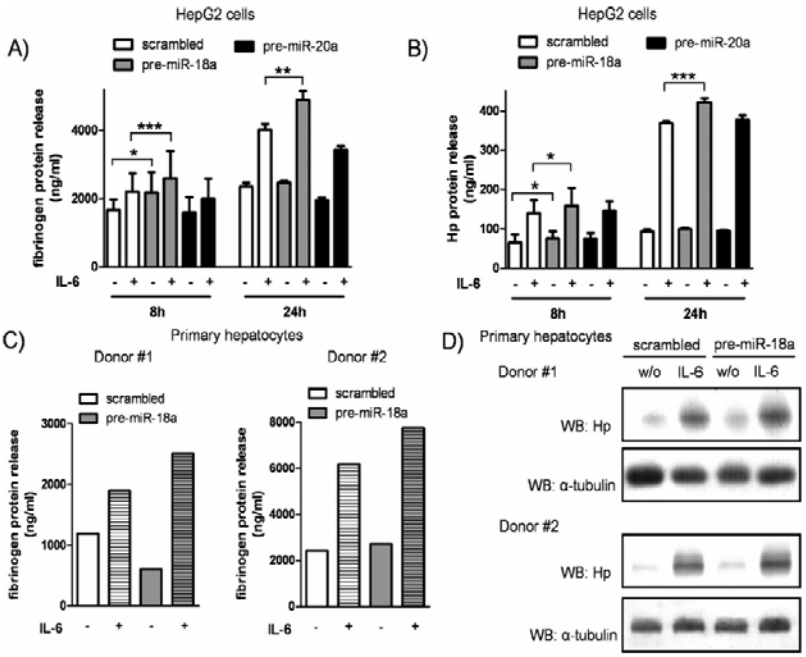

FIGURE 3. **Overexpression of miR-18a promotes expression and release of acute-phase proteins.** The protein levels of fibrinogen and Hp in pre-miRNA-transfected HepG2 cells and hepatocytes were quantified by ELISA or Western blotting. *A*, release of fibrinogen into the supernatants after 8 and 24 h of IL-6 stimulation (20 ng/ml) was significantly increased in cells overexpressing miR-18a as shown by ELISA. miR-20a transfection failed to increase the release of fibrinogen ($n = 5$). *B*, the same supernatants were assessed for quantification of Hp protein levels, showing enhanced secretion of Hp after 8 and 24 h of IL-6 stimulation when miR-18a was overexpressed. Transfection of miR-20a did not change the release of Hp ($n = 5$). *C*, primary hepatocytes were transfected with pre-miR-18a and stimulated with IL-6 (20 ng/ml) for 24 h. The total amount of fibrinogen in supernatants was quantified by ELISA, showing that the IL-6-induced release of fibrinogen was enhanced in hepatocytes transfected with pre-miR-18a ($n = 2$). *D*, the same cells were analyzed for protein levels of Hp by Western blotting (*WB*). The expression of Hp was increased by miR-18a transfection compared with scrambled transfection ($n = 2$). Statistical analysis was by Student's paired *t* test. *w/o*, without. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

To address the question of whether the observed reduction in PIAS3 expression is directly miR-18a-driven, we performed reporter gene studies in HEK293 cells. The entire 3′-UTR of PIAS3 was cloned into the pGL3-Control vector, creating a luciferase reporter system with the respective seed sequence for miR-18a (Fig. 4*D*). As a negative control, the "antisense" construct was generated (15). In addition, we mutated the miR-18a seed match sequence in the "sense" construct by introducing four point mutations (3′-UTR of PIAS3ΔmiR-18a) (Fig. 4*D*). Cotransfection of the PIAS3 3′-UTR WT construct and pre-miR-18a yielded a significantly reduced relative luciferase activity (0.6 ± 0.07-fold, $p < 0.001$) (Fig. 4*E*). The antisense construct, as well as the mutant construct, was not affected by overexpression of miR-18a. These findings imply a direct interaction between the 3′-UTR of PIAS3 and miR-18a.

PIAS3 Is an Important Modulator of the Acute-phase Response—So far, our data demonstrated that miR-18a promotes the acute-phase response and directly inhibits PIAS3, a known repressor of STAT3 activity. Due to the fact that the role of PIAS3 in the acute-phase response was still unknown and to mimic the effects of miR-18a, we silenced PIAS3 and, as an additional control, STAT3 in HepG2 cells. (mRNA levels were reduced by 32 ± 12 and 69 ± 6%, respectively ($p < 0.001$).) Next, we performed reporter gene studies to monitor the promoter activity of Hp and FGG in siRNA-transfected and IL-6-stimulated HepG2 cells. The IL-6-induced promoter activity of Hp was significantly reduced in STAT3-silenced cells (7.43 ± 2.01-fold, $p < 0.001$) compared with scrambled transfection (21.17 ± 3.55-fold) (Fig. 5*A*). Conversely, the response to IL-6 was increased when the expression of PIAS3 was silenced (28.15 ± 3.63-fold, $p = 0.015$). Promoter activity studies of FGG revealed a similar result showing a significant reduction (silencing of STAT3) and increase (silencing of PIAS3) of the IL-6 response in HepG2 cells (Fig. 5*B*). To support these findings further, the mRNA levels of Hp and FGG were measured after 4 h of IL-6 stimulation. Fig. 5*C* summarizes the quantification of Hp mRNA levels, showing an IL-6-induced up-regulation of Hp by 7.32 ± 1.41-fold in scrambled transfected cells. Transfection of STAT3 siRNA reduced the IL-6-induced stimulation of Hp mRNA expression (4.78 ± 0.44-fold, $p < 0.001$); in contrast, the expression of Hp was further increased when PIAS3 was silenced concomitantly (9.65 ± 2.15-fold, $p = 0.015$). A similar expression pattern was detected when the same samples were analyzed for the expression of FGG mRNA (Fig. 5*D*).

miR-18a Promotes Phosphorylation and Expression of STAT3—The effect of miR-18a on the phosphorylation of STAT3 was tested by performing Western blot experiments. As shown in Fig. 6*A*, the IL-6-induced phosphorylation of STAT3 was found to be more pronounced in pre-miR-18a-transfected HepG2 cells (STAT3-P/α-tubulin ratio, 0.84 ± 0.15 (scrambled transfection) and 1.17 ± 0.31 (pre-miR-18a transfection), $p = 0.039$) (Fig. 6*B*). Induction peaked 30 min after IL-6 stimulation, indicating enhanced activity of STAT3. In addition, the expression of STAT3 was increased at the protein level (STAT3/α-tubulin

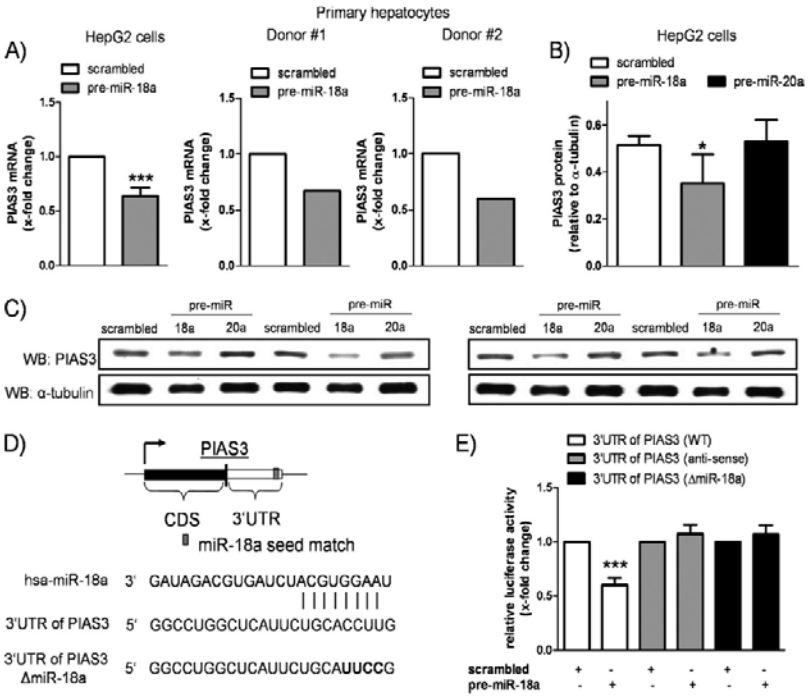

FIGURE 4. **PIAS3 is a direct target of miR-18a.** *A*, miR-18a-transfected HepG2 cells revealed a significant down-regulation of PIAS3 mRNA levels compared with the scrambled control ($n = 7$). The mRNA levels of PIAS3 were also found to be decreased by miR-18a transfection in primary hepatocytes ($n = 2$). *B*, Western blotting confirmed the reduced expression of PIAS3 at the protein level in HepG2 cells as assessed by densitometric analysis ($n = 4$). Specific miR-18a-mediated down-regulation of PIAS3 was proven by transfection of a non-predicted miRNA, miR-20a, showing no alterations in the protein expression of PIAS3. *C*, Western blots (*WB*) used for densitometric analysis are shown ($n = 4$). *D*, TargetScan prediction software identified one seed match of miR-18a in the 3′-UTR of PIAS3. The predicted base pairing of PIAS3 mRNA and miR-18a is shown. The entire 3′-UTR of PIAS3 was used for reporter gene studies, and the predicted miR-18a seed sequence was destroyed by introducing four point mutations. *CDS*, coding sequence; *hsa*, *Homo sapiens*. *E*, reporter gene studies in HEK293 cells showed that cotransfection of miR-18a significantly decreased the relative luciferase activity compared with scrambled transfection. The antisense construct and the mutant construct (ΔmiR-18a) were not affected by overexpression of miR-18a ($n = 7$). Statistical analysis was by Student's paired *t* test (*A* and *E*; scrambled transfection was set to 1) or by Student's unpaired *t* test (*B*). *, $p < 0.05$; ***, $p < 0.001$.

ratio, 0.62 ± 0.13 (scrambled transfection unstimulated) and 1.12 ± 0.26 (pre-miR-18a transfection unstimulated), $p = 0.002$) (Fig. 6*C*) and at the mRNA level (1.68 ± 0.18-fold, $p < 0.001$) (Fig. 6*D*) by pre-miR-18a transfection. Taken together, our data present a novel IL-6/miR-17/92 pathway that enhances the expression and release of the acute-phase proteins fibrinogen and Hp by promoting STAT3 phosphorylation and PIAS3 inhibition, thereby augmenting STAT3 activation.

DISCUSSION

In this study, we have demonstrated a novel regulatory pathway of the acute-phase response. Briefly, we found that IL-6 stimulated the expression of the miRNA cluster miR-17/92 in HepG2 cells and primary hepatocytes, representing a novel positive feedback loop of IL-6 signaling through the repression of the STAT3 inhibitor PIAS3. Thus, overexpression of the miR-17/92-derived miR-18a enhanced the production of the acute-phase proteins fibrinogen and Hp, providing evidence for an important role of miRNAs in the regulation of the inflammatory response.

To a substantial extent, the acute-phase response is controlled by the cytokine IL-6, which is released from monocytes and other immune cells during acute and chronic inflammatory disorders (18). In the liver, the acute-phase response is initiated by the IL-6-induced phosphorylation and dimerization of the transcription factor STAT3, which subsequently shuttles to the nucleus (3). STAT3 homodimers bind to specific DNA sequences in promoter regions and stimulate the transcription of IL-6 target genes (19). We have shown recently that, in human pulmonary arterial endothelial cells, STAT3 directly activates the transcription of miR-17/92 upon stimulation with IL-6 (12). Here, analogous stimulation experiments with IL-6 in HepG2 cells revealed a dose-dependent up-regulation of the primary transcript of miR-17/92 (*i.e. C13orf25*). Together with our previous results (12), the induction of miR-17/92 by IL-6 thus appears to be a STAT3-mediated and cell type-independent mechanism. In this regard, we identified an evolutionarily conserved core palindromic TT-AA motif with a 4-bp spacing in the promoter of miR-17/92, which is responsible for STAT3 binding (12).

To maintain a physiological steady state under normal conditions, the function and expression of STAT3 need to be finely balanced, which is achieved mainly by the concerted action of intracellular feedback loops. We therefore hypothesized that the STAT3-regulated miR-17/92 cluster might be involved in the feedback signaling of STAT3 as well. We thus measured the promoter activity of the STAT3 target genes Hp and FGG in HepG2 cells transfected with miRNA mimics. Overexpression of miR-18a and miR-20a resulted in enhanced activation of

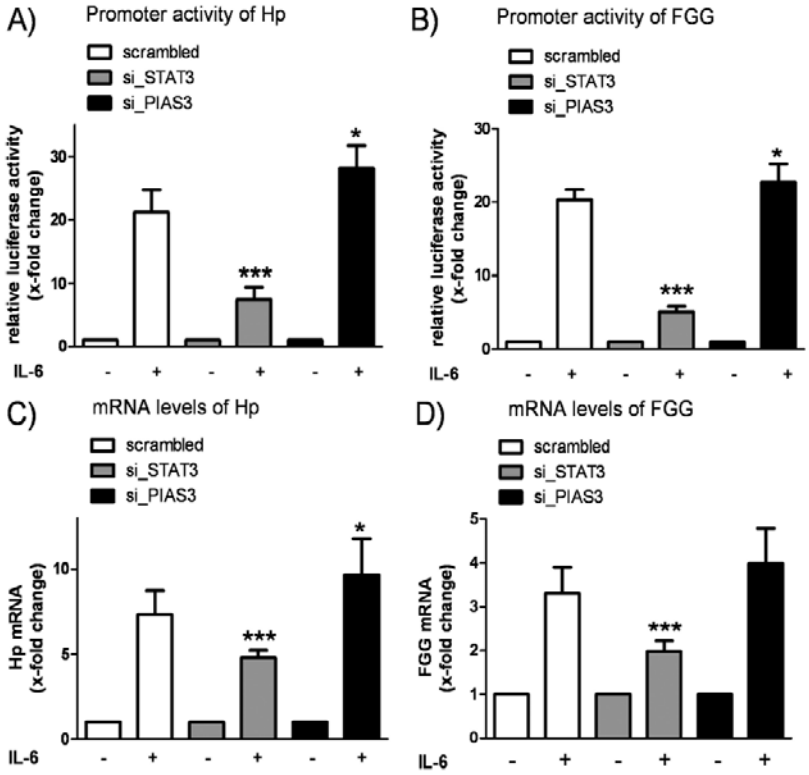

FIGURE 5. **PIAS3 is an important mediator of acute-phase response.** HepG2 cells were transfected with siRNAs targeting PIAS3 (*si_PIAS3*) and STAT3 (*si_STAT3*). *A*, IL-6 stimulation (20 ng/ml) for 4 h activated the promoter of Hp in all samples, with silencing of STAT3 reducing the activity of luciferase compared with scrambled transfection. Conversely, silencing of PIAS3 increased the relative luciferase activity ($n = 5$). *B*, promoter studies of FGG revealed a similar pattern of activation showing a significant reduction (STAT3 siRNA) and enhancement (PIAS3 siRNA) of the IL-6 response in HepG2 cells ($n = 7$). *C*, the mRNA levels of Hp after 4 h of IL-6 stimulation were significantly decreased (STAT3 siRNA) or increased (PIAS3 siRNA) compared with scrambled transfection ($n = 9$). *D*, the same samples were analyzed for expression of FGG mRNA, showing a similar expression pattern ($n = 9$). The respective unstimulated samples were set to 1. Statistical analysis was by Student's unpaired *t* test. *, $p < 0.05$; ***, $p < 0.001$.

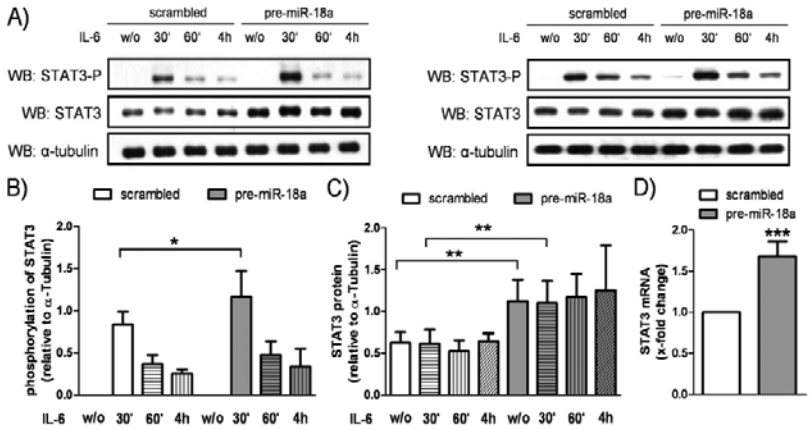

FIGURE 6. **miR-18a promotes phosphorylation and expression of STAT3.** HepG2 cells were transfected with pre-miR-18a or the scrambled negative control, serum-starved, and stimulated with IL-6 (20 ng/ml) for 30 min, 1 h, or 4 h. *A*, Western blot (*WB*) experiments were performed to analyze the phosphorylation and expression of STAT3. Two representative Western blots are shown. *w/o*, without. *B*, phosphorylation of STAT3 (Tyr-705) was markedly enhanced in pre-miR-18a-transfected cells compared with scrambled transfection. Densitometric analysis revealed a significant increase after 30 min of IL-6 stimulation ($n = 6$). *C*, transfection of miR-18a led to a significant up-regulation of the protein levels of STAT3 in IL-6 naïve and IL-6-stimulated HepG2 cells. The results from densitometric analysis are shown ($n = 6$). *D*, the mRNA levels of STAT3 were found to be up-regulated in miR-18a-overexpressing cells ($n = 7$). Statistical analysis was by Student's unpaired *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

STAT3 upon IL-6 stimulation as shown by promoter studies. These results were further confirmed by showing increased mRNA expression levels of Hp and FGG upon transfection of miR-18a and miR-20a, respectively. With respect to these findings we conclude that miR-17/92 acts as an enhancer of STAT3 activity and represents a positive feedback loop in the IL-6 sig-

naling cascade. Interestingly, Pichiorri *et al.* (8) demonstrated that another miRNA encoded by miR-17/92, miR-19a, augments STAT3 activity by targeting SOCS1, a known suppressor of STAT3 phosphorylation. In contrast to these findings, we did not observe increased IL-6 signaling in miR-19a-transfected HepG2 cells. These differences are probably due to the respective experimental settings: because SOCS1 is part of a negative feedback loop of STAT3 activity (6) and thus exerts its function with temporal delay, the 4 h of IL-6 stimulation employed in our study might be too short to observe a miR-19a-mediated effect on STAT3 activity.

Because miRNAs have been associated mainly with the repression of gene expression (7), the observed up-regulation of acute-phase genes upon overexpression of miRNAs was most likely due to an indirect effect (*i.e.* the repression of a yet undefined inhibitor). A computational approach identified PIAS3, which interferes with the DNA-binding activity of STAT3 (17), to contain a potential binding site for miR-18a. Overexpression of miR-18a decreased the expression of PIAS3 at both the mRNA (probably due to mRNA cleavage) and protein levels. Furthermore, by performing reporter gene studies with the 3′-UTR of PIAS3, we confirmed a direct miR-18a-driven repression of PIAS3. We demonstrated the importance of PIAS3 for acute-phase response by showing that knockdown of PIAS3 led to enhanced activation of the promoters of FGG and Hp and thus increased expression of Hp and FGG mRNAs. The correlation between stimulation with IL-6 and expression of *C13orf25* and PIAS3 was further investigated by kinetic experiments and revealed a down-regulation of PIAS3 after long-term stimulation (supplemental Fig. S3).

To determine whether miR-18a also enhances the secretion of acute-phase proteins and to put our findings into a physiological context, we quantified the levels of Hp and fibrinogen in the supernatants of pre-miRNA-transfected HepG2 cells. We found the release of Hp and fibrinogen to be enhanced by miR-18a under both IL-6 naïve and IL-6-stimulated conditions. These findings emphasize the potency of miR-18a as a regulator of the acute-phase response via STAT3 signaling.

Loss-of-function experiments by antagonizing miR-18a to inhibit STAT3 signaling were performed in HepG2 cells transfected with small antisense molecules directed against miR-18a (anti-miR-18a). These experiments showed reduced promoter activity and mRNA expression levels of FGG and Hp and decreased secretion of fibrinogen and Hp in anti-miR-18a-treated cells. On the other hand, the protein levels of PIAS3 were found to be up-regulated under these conditions. These data confirm the physiological role of miR-18a in the IL-6 signaling cascade and further demonstrate that silencing of miR-18a may provide a useful tool to interfere with the hepatic acute-phase response.

The phosphorylation of Tyr-705 of STAT3 is an essential trigger of the acute-phase reaction and thus of special importance (3). Here, we found that Tyr-705 was more phosphorylated in pre-miR-18a-transfected cells, indicating enhanced STAT3 activity. These findings support the conclusion that miR-18a acts as enhancer of the IL-6 pathway. Interestingly, the expression of STAT3 by pre-miR-18a transfection was found to be increased at both the mRNA and protein levels. Because the

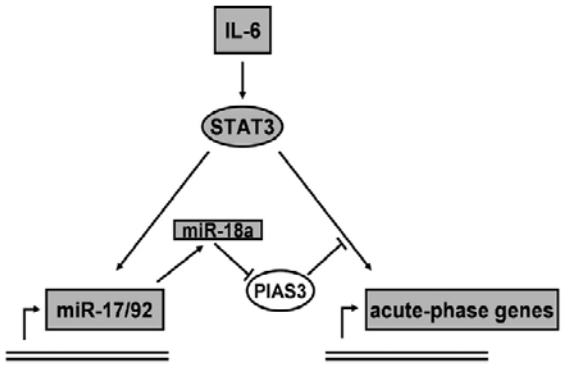

FIGURE 7. **Regulation of acute-phase gene expression by miR-17/92 through a positive feedback loop involving PIAS3.** We propose the following model for the regulation of the acute-phase response by the miR-17/92 cluster. IL-6, released from immune cells, activates STAT3 in hepatocytes, thus triggering the expression of acute-phase genes and the miRNA cluster miR-17/92. In turn, miR-18a targets the STAT3 inhibitor PIAS3, which leads to the enhanced activation of STAT3 and thus results in an increased release of acute-phase proteins.

inhibitory action of PIAS3 affects the DNA-binding activity of STAT3, one might propose an additional, PIAS3-independent effect of miR-18a on Tyr-705 phosphorylation. Whether this effect is due to increased expression of the unphosphorylated STAT3 substrate remains unclear and needs to be addressed by further studies.

In addition to miR-18a, the STAT3-dependent activation of the Hp and FGG promoters was significantly enhanced when HepG2 cells were transfected with precursor molecules of miR-20a. ELISAs for Hp and fibrinogen did not reveal, however, an elevated release of these proteins when miR-20a was overexpressed. Because two independent methods (reporter gene assay and quantitative real-time PCR) showed an increase in STAT3 activity by miR-20a, we suggest that the failure of increased acute-phase protein secretion by miR-20a might be due to an impaired protein release, or, alternatively, that miR-20a targets not only repressors but also activators of the IL-6 transduction pathway, *e.g.* JAK1 (Janus kinase 1). JAK1 is the kinase responsible for STAT3 phosphorylation and is also targeted by miR-20a (20).

The most important findings of this study were confirmed by performing experiments in an additional cell model using primary human hepatocytes. By overexpressing miR-18a in these cells, we could show that miR-18a augments the IL-6-induced acute-phase response by targeting the repressor of IL-6 signaling, PIAS3. Similarly, IL-6 treatment increased the expression of miRNAs derived from the miR-17/92 cluster, suggesting the presence of a positive feedback loop in both primary hepatocytes and HepG2 cells.

In summary, we propose the following model for the regulation of the acute-phase response by miR-17/92 (Fig. 7). During states of acute or chronic inflammation, immune cells secrete IL-6, which causes the activation of STAT3 in hepatocytes. This triggers the production of acute-phase proteins and their systemic release. At the same time, the expression of miRNAs derived from the cluster miR-17/92 is up-regulated. This leads to a further augmentation of STAT3 activity through the

repression of PIAS3 by miR-18a and finally contributes to the overwhelming release of acute-phase proteins.

In conclusion, we identified here a novel IL-6/miR-17/92 pathway that, through the inhibition of PIAS3, enhances the production and release of the acute-phase proteins fibrinogen and Hp. To our knowledge, this is the first report on a miRNA-mediated regulation of PIAS3, thus contributing to the understanding of the complex regulatory mechanisms within the signal transduction of IL-6.

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Supplementary information

Expanded materials and methods

Cell culture

Human hepatocellular carcinoma (HepG2) cells and human embryonic kidney (HEK)293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen AG, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS; Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2. Human primary hepatocytes (n=2) were purchased from Lonza (Lonza, Verviers, Belgium) and cultured according to the manufacturer's instructions. One donor was found to have metastatic liver disease with altered cellular response and was excluded from analysis; cells were kindly replaced with a novel batch of hepatocytes by Lonza. Prior to IL-6 stimulation, HepG2 cells were serum starved for period of 24h (0.5% FCS). IL-6 (R&D Systems, Abingdon, United Kingdom) was used as indicated.

Quantitative real time-PCR (qPCR) analysis

Total RNA was extracted using the RNeasy kit (Qiagen AG, Hombrechtikon, Switzerland). RNA was reverse transcribed by using Random Hexamers and MultiScribe Reverse Transcriptase (both from Applied Biosystems, Rotkreuz, Switzerland). Quantification of specific RNA transcripts was performed by SYBR Green qPCR, using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Sequences of primers used for amplification of human fibrinogen-γ-chain (FGG), human haptoglobin (Hp), human primary transcript of the miR-17/92 cluster (C13orf25), human signal transducer and activator of transcription (STAT)3, human protein inhibitor of activated STAT (PIAS)3 are shown in Suppl. Table S1. To confirm specific amplification, a dissociation curve analysis was performed. The amounts of loaded RNA were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or hypoxanthine phosphoribosyltransferase 1 (HPRT1). Differential gene expression was calculated with the threshold cycle (C_t) method (1).

Quantitative real time-PCR (qPCR) analysis

Total RNA was extracted using the mirVana (Applied Biosystems) or the miRNeasy miRNA isolation kit (Qiagen). Mature microRNA sequences were detected by specific stem-loop

primers, reverse transcribed using MultiScribe Reverse Transcriptase and quantified by performing SYBR Green qPCR (2). Stem-loop primers and amplification primers were designed for miR-18a, miR-19a, miR-20a, and miR-92a (Suppl. Table S1). Obtained signals were normalized to the expression of GAPDH or RNU48 and specific amplification was confirmed by performing dissociation curve analysis.

Western blot

For protein extraction cells were lysed with sample loading buffer. Whole-cell lysates were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred to nitrocellulose membrane. Membranes were blocked with dry milk and incubated with the following primary antibodies: anti – PIAS3, anti – Hp (both from Abcam, Cambridge, UK), anti - STAT3 (R&D Systems), anti – Phospho-STAT3 (Tyr705, Cell Signaling Technology, Danvers MA, USA), and anti – α-tubulin (Sigma, St. Louis, MO, USA). Bands were detected with species-specific secondary antibodies coupled to horseradish peroxidase (Jackson ImmunoResearch Europe Ltd, Suffolk, UK). Evaluation of the expression of proteins was performed by the Alpha Imager software (Alpha Innotech, San Leandro, CA, USA) via pixel quantification of the electronic image.

Plasmid construction

For promoter studies, a fragment (878 bp) of the Hp promoter containing two STAT3 binding sites was amplified from human genomic DNA (Promega AG, Dübendorf, Switzerland) using PCR. The PCR product was digested with *Bgl*II and *Hind*III and cloned into the firefly luciferase-based pGL3basic vector (Promega). The same cloning strategy was applied for the cloning of the promoter of FGG (578 bp, containing one STAT3 binding site) into the pGL3basic vector. For normalization of the luciferase activity, a modified vector encoding for *Renilla* luciferase (pRL-SV40, Promega) was used. The SV40 promoter of pRL-SV40 was removed by digestion with *Bgl*II and *Hind*III and replaced by the promoter of GAPDH (1063 bp). Primer sequences used for cloning are shown in Suppl. Table S1. The correct sequence of each insert was confirmed by sequencing.

For confirmation of direct miRNA - mRNA interactions, the 3'untranslated region (UTR) of PIAS3 (924 bp) was amplified from human genomic DNA using PCR. The obtained PCR product was digested with *Nhe*I and cloned into the pGL3control vector (Promega). As a negative control, the anti-sense construct was used (3). In addition, the corresponding miRNA seed match located in the 3' UTR of PIAS3 was specifically mutated by using mutagenesis

PCR. The correct sequences and orientation of the inserts were confirmed by sequencing. The primer sequences are provided in Suppl. Table S1.

Reporter gene assay

For promoter activity studies, HepG2 cells were transfected either with pGL3basic-Hp or with pGL3basic-FGG (600ng/well) using Lipofectamine 2000 (Invitrogen). In addition, a vector for normalization (pRL-GAPDH, 300ng/well) was co-transfected. After 24h, the cells were stimulated with IL-6 (20ng/ml) for 4h, harvested, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The values obtained were normalized to the activity of *Renilla* luciferase (pRL-GAPDH).

For miRNA target validation, HEK293 cells were transfected with the pGL3control 3‘UTR constructs of PIAS3 (150ng/well) using Lipofectamine 2000. Moreover, a vector for normalization (pRL-SV40, 80ng/well) and precursor molecules of miRNAs (negative control#1 or pre-miR-18a, both from Applied Biosystems/Ambion; 25nM) were added. After 24h, the cells were lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System. The values obtained were normalized to the activity of *Renilla* luciferase (pRL-SV40).

Transfection of miRNAs

For specific over expression of miRNAs in HepG2 cells precursor molecules of miRNAs were purchased from Applied Biosystems/Ambion (pre-miR-18a, -19a, -20a, -92a). Scrambled negative control #1 served as scrambled control. For over expression of the entire cluster (cocktail), 25nM of each miRNA was transfected. In addition, 100nM of each miRNA was used in single transfection studies. Nuclear transfection of HepG2 (100nM of RNA) was achieved by using the cell line nucleofector kit V (Amaxa GmbH, Cologne, Germany). Alternatively, HepG2 cells and primary hepatocytes were transfected with the same amount of pre-miRNAs using Lipofectamine 2000. For specific silencing of miR-18a HepG2 cells were transfected with anti-miR-18a (100nM, Applied Biosystems/Ambion) using cell line nucleofector kit V or Lipofectamine 2000. Following an incubation period of 72h, cells were stimulated with IL-6 (20ng/ml) for 4h, harvested, and gene expression analysis was performed. In addition, promoter activity studies in miRNA over expressing HepG2 cells were performed.

Gene silencing

HepG2 cells were transfected with 200nM of small interfering RNA molecules (siRNAs, all provided by Qiagen) using cell line nucleofector kit V (AMAXA). AllStars negative control siRNA (Qiagen) served as scrambled control. After an incubation period of 48h, cells were stimulated with IL-6 (20ng/ml) for 4h, harvested, and gene expression was analyzed. Furthermore, promoter studies in siRNA-transfected HepG2 cells were performed.

Enzyme-linked immunosorbent assay (ELISA)

HepG2 cells and primary hepatocytes were transfected with pre-miRNAs or anti-miRNAs (100nM) using Lipofectamine 2000. Following an incubation period of 72h, cells were stimulated with IL-6 (20ng/ml) for 8h or 24h and supernatants were collected. Quantitative determination of human Hp and FGG was achieved by using commercially available ELISA kits (GenWay Biotec Inc, San Diego, CA, USA) according to the manufacturer's instructions.

Supplementary figures and table

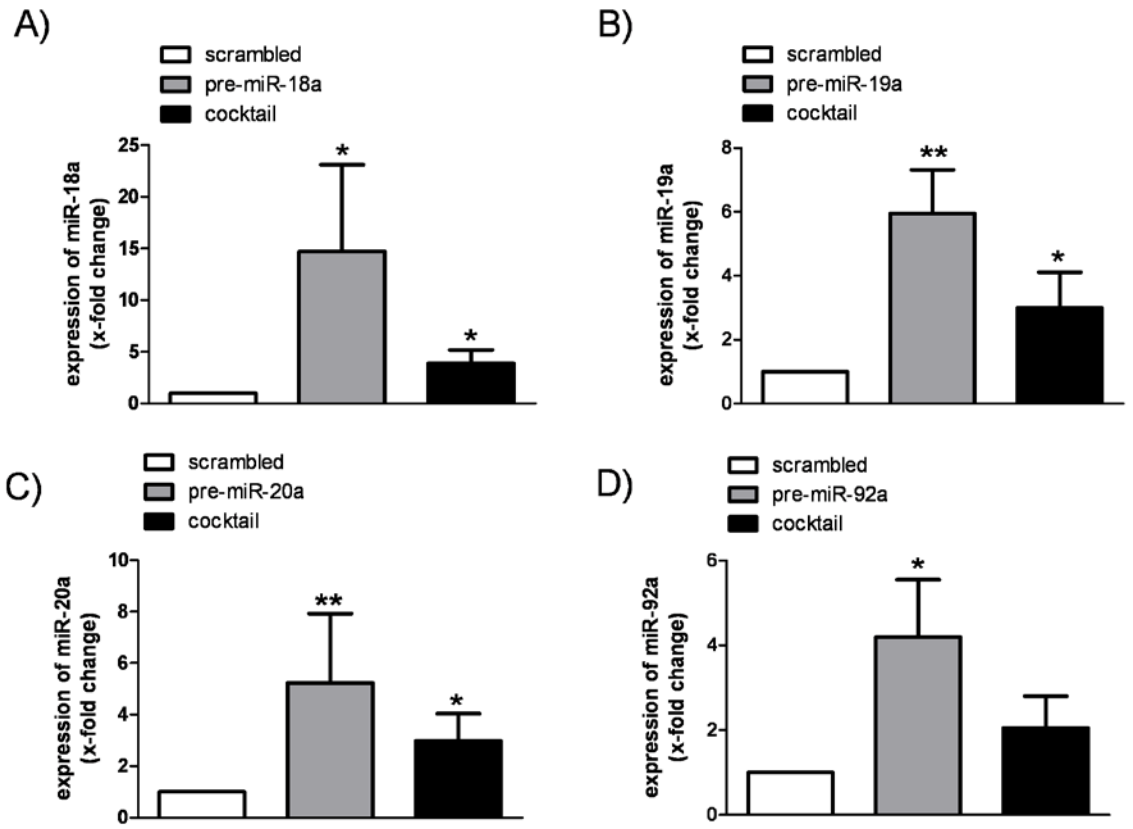

Figure S1. Over expression of miR-17/92 in transfected HepG2 cells.

HepG2 cells were transfected with miRNA precursor molecules (pre-miR-18a, -19a, -20a, -92a, and a cocktail of all) or scrambled negative control. (A) Stem-loop qPCR was used to quantify levels of miR-18a showing a significant upregulation of miR-18a in pre-miR-18a and cocktail transfected HepG2 cells (n=4). The same method was applied for quantification of miR-19a (B), miR-20a (C), and miR-92a (D) in pre-miRNA transfected HepG2 cells confirming a significant increase of miRNA expression, respectively (n=4). The respective scrambled transfection was set to 1. Statistical analysis by paired student's t-test.

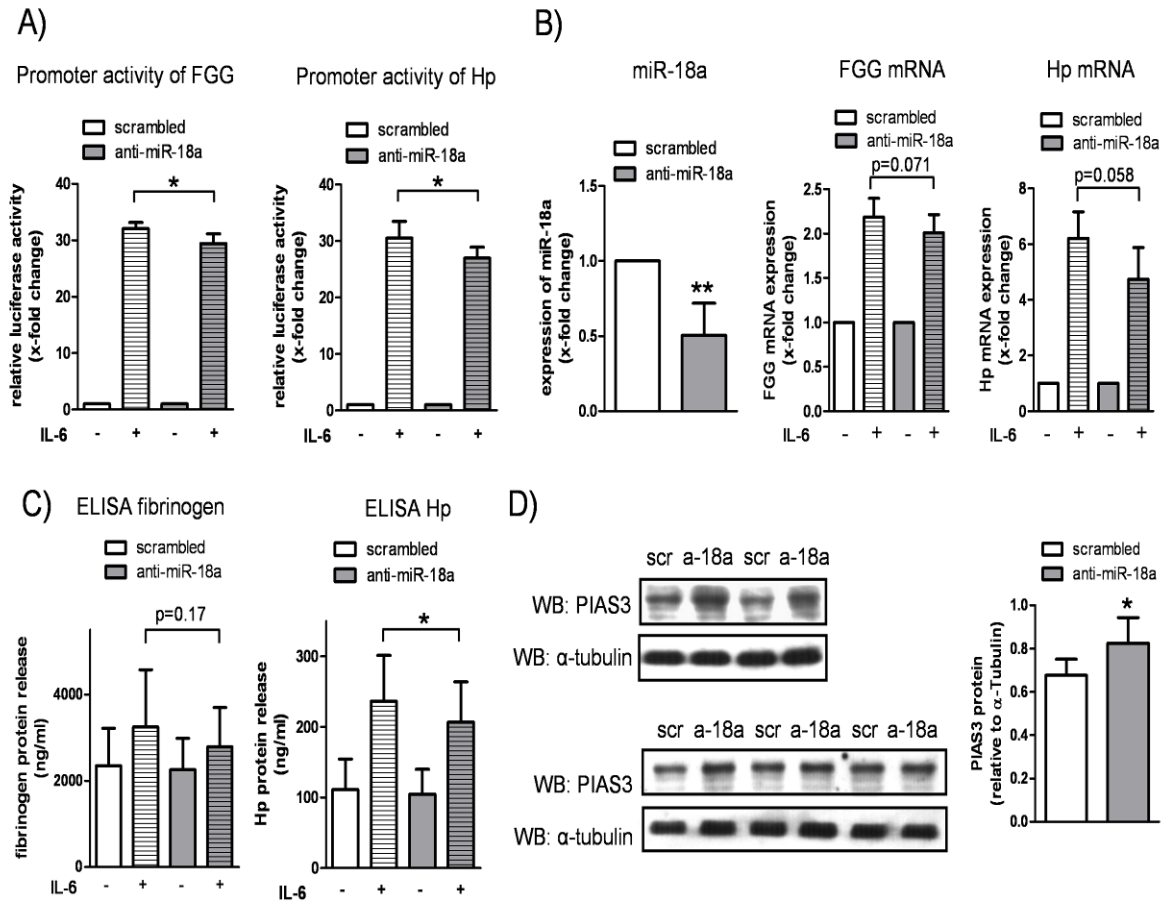

Figure S2. Silencing of miR-18a decreases acute-phase response in HepG2 cells.

Antagonizing miR-18a expression was achieved by transfection of HepG2 cells with anti-miR-18a molecules. (A) Promoter activity of FGG and Hp was assessed by co-transfection of a luciferase-based vector system comprising promoter sequence of either FGG or Hp. IL-6 stimulation (20ng/ml) for 4h activated the promoter of FGG and Hp in all samples with the silencing of miR-18a reducing the activity of luciferase as compared to scrambled transfection (n=6). (B) Stem-loop qPCR was used to quantify levels of miR-18a showing a significant downregulation of miR-18a in anti-miR-18a

transfected HepG2 cells (n=6). mRNA levels of FGG and Hp after 4h of IL-6 stimulation (20ng/ml) were found to be reduced in anti-miR-18a treated cells compared to scrambled transfection (n≥5). (C) Protein levels of fibrinogen and Hp in the supernatants of anti-miR-18a transfected HepG2 cells were quantified by ELISA. Release of fibrinogen after 8h of IL-6 stimulation (20ng/ml) was decreased in cells transfected with anti-miR-18a as compared to scrambled transfection. The same supernatants were assessed for quantification of Hp protein levels and showed significantly decreased secretion of Hp when miR-18a was blocked (n=6). (D) Western blot experiments showed increased expression of PIAS3 protein levels in anti-miR-18a transfected HepG2 cells confirming the regulation of PIAS3 by miR-18a (n=5). Statistical analysis was performed by using the student's t-test (unpaired for A, B, D and paired for C).

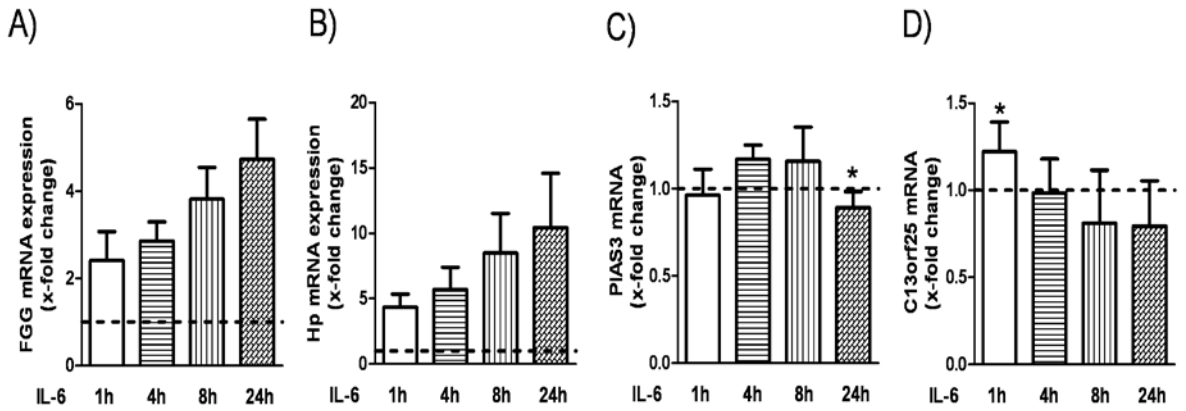

Figure S3. Expression of PIAS3 is reduced after IL-6 stimulation.

RNA levels of FGG, Hp, PIAS3, and C13orf25 were measured in HepG2 cells stimulated with IL-6 (20ng/ml). (A) mRNA levels of FGG and (B) Hp were found to be enhanced in a time-dependent manner by IL-6 stimulation, whereas the expression of (C) PIAS3 was significantly downregulated at 24h of IL-6 treatment (0.89±0.09 fold, p=0.036) (n≥5). (D) The expression of the primary transcript of miR-17/92 (C13orf25) was significantly induced at 1h of IL-6 treatment (1.22±0.17 fold, p=0.001) (n≥5). The correlation of PIAS3 and C13orf25 is deferred probably due to the miRNA maturation process (4). The dashed line indicates unstimulated control and was set as 1. Statistical analysis by paired student's t-test.

Table S1. Primer sequences

gene expression analysis	
GAPDH fwd	5’ – GGG AAG CTT GTC ATC AAT GGA – 3’
GAPDH rev	5’ – TCT CGC TCC TGG AAG ATG GT – 3’
HPRT1 fwd	5’ – ATG GAC AGG ACT GAA CGT CTT G – 3’
HPRT1 rev	5’ – GGC TAC AAT GTG ATG GCC TC – 3’
FGG fwd	5’ – TAT TAC CAA GGT GGC ACT TAC TC – 3’
FGG rev	5’ – CAT TAT CAT AAC CAT TAG GAG TAG ATG – 3’
Hp fwd	5’ – TCA CGG ATA TCG CAG ATG ACG – 3’
Hp rev	5’ – CAC ATA GCC ATG TGC AAT CTC G – 3’
C13orf25 fwd	5' – TTG CTA AGT GGA AGC CAG AAG – 3'
C13orf25 rev	5' – CAT CCA CGT GGC AAA ACA T – 3'
PIAS3 fwd	5' – GAT TCT TAC ATC CAG AGA GGT TC – 3'
PIAS3 rev	5' – ACC TGT ATG GTA TAA TCA CAT TTG G – 3'
STAT3 for	5' – TTC ACT TGG GTG GAG AAG GAC A – 3'
STAT3 rev	5' – CGG ACT GGA TCT GGG TCT TAC C – 3'
miRNA expression analysis	
miR-18a RT (stem loop primer)	5’ – GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TAT CT – 3’
miR-18a fwd	5’ – GGC GGT AAG GTG CAT CTA GT – 3’
miR-18a rev	5’ – GTG CAG GGT CCG AGG T – 3’
miR-19a RT (stem loop primer)	5’ – GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAG TT – 3’
miR-19a fwd	5’ – CGG CGG TGT GCA AAT CTA TGC – 3’
miR-19a rev	5’ – GTG CAG GGT CCG AGG T – 3’
miR-20a RT (stem loop primer)	5’ – GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT ACC TG – 3’
miR-20a fwd	5’ – GCG GCG GTA AAG TGC TTA TAG TG – 3’
miR-20a rev	5’ – TGC AGG GTC CGA GGT AT – 3’

miR-92a RT (stem loop primer)	5’ – GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CAG GC – 3’
miR-92a fwd	5’ – CGG CGG TAT TGC ACT TGT CCC – 3’
miR-92a rev	5’ – GTG CAG GGT CCG AGG T – 3’
RNU48 RT (stem loop primer)	5’ – GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GTC AG – 3’
RNU48 fwd	5’ – CCA TGA GTG TGT CGC TGA TG – 3’
RNU48 rev	5’ – GTG CAG GGT CCG AGG T – 3’
plasmid construction	
FGG promoter fwd	5’ – GGG CTC GAG ATC TTG AGA AGT GAG AGC CTA TG – 3’
FGG promoter rev	5’ – AAT GCC AAG CTT TGT AAG CTC CTG GGA TAG – 3’
Hp promoter fwd	5’ – GGG CTC GAG ATC TGT TGA TGG GCA TTT GTC TTG – 3’
Hp promoter rev	5’ – AAT GCC AAG CTT GTT GGT CTT GCC TCT GG – 3’
GAPDH promoter fwd	5’ – GGG CTC GAG ATC TAC CTG TTC CCA CCG TGT G – 3’
GAPDH promoter rev	5’ – AAT GCC AAG CTT AGG CGG TGA CTC GGA CC – 3’
3'UTR of PIAS3 fwd	5’ – TAA TGC TAG CGT TCC CTG GAT TAT GGA AAC – 3’
3'UTR of PIAS3 rev	5’ – CGA CGC TAG CGA ACA TTC ACA ACC TTT ATT ATG – 3’
3'UTR of PIAS3 mut fwd	5’ – CTG CAT TGG TCC AGG GAG GTG GAA G – 3’
3'UTR of PIAS3 mut rev	5’ – CTG GAC CAA TGC AGA ATG AGC CAG G – 3’

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5. Discussion and outlook

5.1. The regulation of BMPR2 by microRNAs

In the first part of this thesis, we found that (i) the expression of BMPR2 is regulated by the miR-17/92 cluster on a post-transcriptional level; (ii) this effect is mainly driven by miR-20a; (iii) IL-6 increases the expression of miR-17/92 in HPAECs; (iv) the promoter region of C13orf25 contains a functional STAT3 binding site; and, finally, (v) persistent activation of STAT3 leads to increased expression of miR-17/92, which, in turn, reduces the expression of BMPR2.

The loss of BMPR2 expression is a common feature of PH (Morrell, 2010) and it is believed that the observed change in the proliferation of SMCs which promotes the vascular remodeling in PH is a consequence of the reduced BMPR2 expression (Yang et al., 2005). However, the molecular mechanisms leading to this downregulation of BMPR2 expression have not been unraveled so far. Previous studies, however, suggested a post-transcriptional mechanism of regulation and, in particular, the contribution of miRNAs. For identification of miRNAs potentially involved in the regulation of BMPR2 we performed a computational screening revealing multiple miRNA binding sites in the 3'UTR of BMPR2. Because of a strong prediction indicated by a high degree of complementarity of the miRNA and its target sequence, we decided to investigate whether miR-17/92 could regulate BMPR2 gene expression. Indeed, we found a phylogenetically conserved signaling mechanism involving IL-6 and miR-17/92 that could provide evidence for a common pathway in the pathogenesis of various distinct forms of PH, all of them characterized by loss of BMPR2 expression.

In animal models of PH the expression of miR-17/92 was found to be significantly increased (Caruso *et al*., 2010), and reduced expression of BMPR2 was described in the same animal models (Takahashi *et al*., 2006; Morty *et al*., 2007). Consequently, these findings support our hypothesis of a miR-17/92-mediated downregulation of BMPR2 in PH. The expression levels of miR-17/92 in patients with PH, however, have not been addressed so far. Future work is needed to investigate whether the expression of miR-17/92 is dysregulated in the pulmonary endothelium of patients suffering from PH. In this regard, it would be interesting to analyze miR-17/92 levels in serum samples and its potential as a biomarker for onset and severity of PH. Since the screening also revealed other miRNAs as potential regulators of BMPR2, including miR-21, miR-25 and miR-153, one might focus further studies on several miRNAs that could potentially be employed as biomarkers of this disease.

In our study we provided evidence that BMPR2 is regulated by miR-17/92. Consistent with these data, TGFBR2, another receptor belonging to the TGF-β superfamily, is also regulated by the miR-17/92 cluster (Dews *et al*., 2010). TGFBR2 mediates apoptosis of ECs and SMCs and has been associated with the pathogenesis of PH (Eickelberg and Morty, 2007). Therefore, miR-17/92 appears to act as a potent repressor of TGF-β and BMP signaling and, thus, might contribute to altered apoptosis resistance and, eventually, to the remodeling of pulmonary arterial vessels.

In our study, the impact of IL-6 on the expression of miR-17/92 was also addressed revealing increased expression of miR-17/92 upon IL-6 stimulation. In addition, we identified a functional STAT3 binding site in the promoter of miR-17/92. STAT3 is the major mediator of IL-6 signaling and was found to be persistently activated in plexiform lesions in severe PH (Masri *et al*., 2007). By transfecting a constitutively active form of STAT3 we found increased levels of miR-17/92 and decreased expression of BMPR2 demonstrating a functional link of STAT3 signaling and the expression of BMPR2. Thus, our study shed new light on the regulation of BMPR2 by inflammatory signals.

In summary, this was the first time that miRNAs have been associated with PH and, moreover, our findings offered for the first time a mechanistic explanation for the downregulation of BMPR2 which has been repeatedly described as important feature in the pathogenesis of PH.

5.2. The role of miR-20a in an *in vivo* model of PAH

Since we assessed the miR-17/92-mediated regulation of BMPR2 in an *in vitro* model without conclusions on the functional relevance of this pathway *in vivo*, we employed an established animal model of PH (exposure to chronic hypoxia) to investigate the physiological role of miR-20a in the second part of this thesis. Briefly, we found that (i) intraperitoneal injections of antagomiR-20a are a feasible approach to efficiently silence the expression of miR-20a in target tissues in a non-toxic manner; (ii) antagonization of miR-20a reduces the hypoxia-induced remodeling of pulmonary vessels and, subsequently, reduces right heart hypertrophy in hypoxic mice; and, (iii) treatment with antagomiR-20a restores expression levels of BMPR2 in lung tissues of hypoxic animals.

Vascular remodeling and occlusion of vessels is commonly observed in pulmonary hypertension and results from vasoconstriction and proliferation of the intimal and medial layer (Humbert *et al*., 2004a). By antagonization of miR-20a vascular remodeling in hypoxic animals was significantly decreased as assessed by wall thickness and occlusion of small

pulmonary vessels. Narrowing of the pulmonary arterial lumen increases vascular resistance that results in cardiac hypertrophy and, ultimately, right heart failure. AntagomiR-20a treated mice revealed significantly less right ventricular hypertrophy as compared to the mock-treated hypoxic control group indicating reduced vascular resistance. Consequently, this study showed that miR-20a contributes to the development of hypoxia-induced vascular remodeling of the small pulmonary arteries. We showed that, by treatment with antagomiR-20a, the development of PH can be prevented. However, it remains unclear at the moment whether the application of antagomiR-20a can also reverse established disease.

Exposure to chronic hypoxia was found to reduce the expression levels of BMPR2 (Takahashi *et al*., 2006). Consistently, we demonstrated that hypoxia decreased mRNA expression of BMPR2 in the mock-treated mice. Of interest, the expression of BMPR2 under hypoxic conditions could be restored and normalized to the levels measured in normoxic control mice by antagonizing miR-20a. These data indicated that miR-20a is an endogenously expressed repressor of BMPR2 expression and, thus, further support our hypothesis of a miR-17/92-mediated regulation of BMPR2. In this regard, it would be of interest to analyze the expression of other validated miR-17/92 targets such as TGFBR2 in order to underpin the role of miR-17/92 as a master regulator of TGF-β and BMP signaling pathway. Moreover, since we showed a functional link of STAT3 and the expression of BMPR2, investigating the influence of antagomiR-20a on the expression and activity of STAT3 would be of particular interest.

An attractive hypothesis is that vascular remodeling might be driven by dysregulated proliferation and apoptosis resistance (Humbert *et al*., 2004a). Since we found less vascular remodeling in mice treated with antagomiR-20a, we assessed proliferation of HPASMC *in vitro* and found that antagomiR-20a significantly reduced their viability. We suggest that the observed decrease in proliferation is caused by two mechanisms. First, it was shown that antagonization of miR-20a leads to upregulation of the cell growth inhibitor p21 (Inomata *et al*., 2009), and, second, the activation of the BMP signaling pathway, which is enhanced upon transfection of antagomiR-20a, was shown to inhibit proliferation of SMCs (Wong *et al*., 2003). Whether silencing of miR-20a can also alter apoptosis or growth of other cells involved in the remodeling of the small pulmonary arteries (e. g. ECs) has to be elucidated by further studies.

Taken together, we reported that downregulation of BMPR2 gene expression and the development of hypoxia-induced changes of small pulmonary arteries can be prevented by the application of antagomiR-20a.

5.3. miR-17/92 signaling in acute-phase response

In the last part of this PhD thesis, we could show that miR-17/92 comprise a novel positive feedback loop of the IL-6 – STAT3 signaling cascade. Briefly, we found that IL-6 stimulated the expression of miR-17/92 in HepG2 cells and primary hepatocytes and that overexpression of one member of miR-17/92, namely miR-18a, significantly increased the IL-6-induced response in these cells. On the molecular level, we identified the STAT3 inhibitor PIAS3 as a novel direct target of miR-18a.

Based on the findings of our previous work, we suggested that miR-17/92 is an important player in the IL-6 signaling cascade and might contribute to the regulation of STAT3 activity. By employing STAT3-inducible reporter gene assays we demonstrated that miR-18a significantly increased the transcriptional activity of STAT3. These findings were further confirmed by showing an enhanced IL-6-induced acute-phase response in hepatocytes upon transfection of miR-18a. Since miRNAs exclusively act as repressors of gene expression (Bartel, 2004), we hypothesized that miR-18a represses a negative regulator of IL-6 signaling. Consequently, we identified PIAS3, which interferes with the DNA binding activity of STAT3 (Chung *et al*., 1997), as a novel direct target of miR-18a.

In addition, overexpression of miR-18a also led to increased expression and phosphorylation of STAT3. Since PIAS3 blocks the DNA binding of STAT3, we suggested a PIAS3-independent and additional effect of miR-18a on STAT3. However, the precise mechanism leading to increased expression and activity of STAT3 by miR-18a remains unclear at the moment. In this regard, we also found increased expression of STAT3 in miR-20a-transfected HepG2 cells (unpublished data) suggesting that miR-20a as well as miR-18a is a potent regulator of STAT3 gene expression. However, miR-20a failed to stimulate the release of acute-phase proteins from IL-6 stimulated HepG2 cells. We suggest that the observed failure of increased acute-phase protein secretion by miR-20a is due to the fact that miR-20a might target not only repressors but also activators of the IL-6 signaling cascade, for example JAK1. JAK1 is a receptor-associated kinase that phosphorylates and activates STAT3 and is a direct target of miR-20a (Doebele *et al*., 2010).

In conclusion, we identified a novel IL-6 – miR-17/92 pathway, which, through the miR-18a-mediated inhibition of PIAS3, enhances IL-6 signaling and, furthermore, represents a novel positive feedback loop of this pathway.

5.4. Experimental questions (follow-up projects)

Based on these studies, the following questions are raised and should be addressed by further experiments:

i) which miRNAs have the potency to be used as biomarkers to distinguish onset, severity and course of human pulmonary hypertension?

ii) do these miRNAs have pathogenetic, diagnostic and/ or therapeutic relevance?

iii) does the inhibition of miRNAs result in a reversal of the vascular remodeling of pulmonary arterial vessels?

iv) is the application of miRNAs a feasible clinical approach to deliver and to influence targeted cells?

v) of what significance are miRNAs in the interplay between inflammation and vascular remodeling with special focus on STAT3 and its miRNA-mediated feedback loops?

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7. Abbreviations

BMP	Bone morphogenetic protein
BMPR2	BMP receptor type II
ECs	Endothelial cells
ELISA	Enzyme-linked immunosorbent assay
FGG	Fibrinogen gamma chain
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HEK293 cells	Human embryonic kidney cells
HepG2 cells	Human hepatoma cells
Hp	Haptoglobin
HPRT1	Hypoxanthine phosphoribosyltransferase 1
Id-1 / -2	Inhibitor of DNA binding 1 / -2
IL-6	Interleukin-6
JAK	Janus kinase
miRNA(s)	microRNA(s)
P(A)H	Pulmonary (arterial) hypertension
PIAS3	Protein inhibitor of activated STAT, 3
qPCR	quantitative polymerase chain reaction
RT-PCR	Reverse transcription-polymerase chain reaction
SOCS	Suppressor of cytokine signaling
STAT3	Signal transducer and activator of transcription 3
SMCs	Smooth muscle cells
TGF-β	Tumor growth factor β
TGFBR2	TGF-β receptor type II

8. Curriculum vitae

Surname: Brock
First Name: Matthias
Date of Birth: 1st October 1982
Birthplace: Germany

Education:

1999-2002	University-entrance diploma (Abitur), secondary school of Hohenmölsen (Gymnasium), Hohenmölsen, Germany
2002-2007	Diploma in Biochemistry, University of Leipzig, Leipzig, Germany
2007-2012	Dr. sc. nat., University of Zurich, Zurich, Switzerland

Publications (original peer reviewed publications)

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Reviews

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